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EFFECT OF INDUCED HYPER- AND HYPOTHYROIDISM ON EXPERIMENTAL ATHEROSCLEROSIS IN COCKERELS

WITH SPECIAL REFERENCE TO THE RELATIONSHIP BETWEEN ACID MUCOPOLYSACCHARIDES AND ATHEROSCLEROSIS

BY

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PREFACE

The present work was carried out at the Department of Forensic Medicine, University of Helsinki in 1957—1960.

The subject of the investigation was suggested to me by Professor U. Uotila, M.D., Chief of the Department of Forensic Medicine, University of Helsinki. Professor Uotila followed closely the progress of my work, giving me guidance and advice without stint of his time. I wish to express my great gratitude to him for all this.

Professor P. I. Halonen, M.D., Head of the Wihuri Research Institute and Chief of the First Medical Clinic, University of Helsinki, read my manuscript. I thank them for their advice and criticism.

In the performance of the biochemical analyses I received advice and assistance from Miss E. Levonen, M. Sc. Miss E. Kaprio and Mrs. L. Schwanck prepared the histological specimens. Miss E. Kivinen looked after the test animals and helped in their preparation. Mr. P. Korhonen took the microscopic photographs at the Institute of Photography, University of Helsinki. My thanks are due to all of them.

Mr. S. Gustavsson, M. Sc., performed the statistical treatment ot the material. Miss P. Ojansuu, M. A., and Mr. L. A. Keyworth, M. A. (Cantab.), translated the manuscript into English, and Assistant Professor T. Mustanoja checked it. I thank them for valuable help.

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Helsinki, January 1961.

Yrjö Perttala

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INTRODUCTION

CONSIDERATIONS PROMPTING THE PRESENT INVESTIGATION

The etiological factors of atherosclerosis can be classified into two groups, general and local. The former include the cholesterol metabolism, the general intermediary metabolism and the factors regulating them. The latter consist of changes occurring in the arterial wall itself.

Dietary fats and their effect on the concentration and quality of the blood cholesterol have been the primary concern of atherosclerosis research of the last few years. Today, however, there is a tendency to concentrate on the elucidation of cholesterol metabolism and intermediary metabolism in general. Special attention is focused on several hormones and certain vitamins which have been found to be of significance in atherogenesis. Among these agents may be mentioned the thyroid hormone, sex hormones, insulin, adrenocortical hormones and vitamins of groups B and C.

As far as local factors are concerned, the theory of passive infiltration has been more or less abandoned and the accumulation of fats in the vascular wall is regarded as a complex chain of events (McMillan *et al.* 1954). The wall of the blood vessel is in fact understood as an active organ whose function and mode of reaction are influenced in many ways (Holman *et al.* 1959). The role of the connective tissue of the vascular wall in atherogenesis has been emphasised frequently.

A significant correlation between goitre and coronary artery mortality was established by Uotila *et al.* (1958) in an autopsy material of the Department of Forensic Medicine, University of Helsinki. They also suggested that there is a connection between the earlier observations that East Finland has a high rate of mor-

tality from coronary disease (Keys *et al.* 1958) and the high incidence of endemic goitre (Wahlberg 1938) which is hypoepithelial in Finland in 48 per cent of the cases (Saarenmaa 1956). According to the hypothesis put forward by Uotila and his co-workers, hypothyroidism and atherosclerosis may in such cases show a causal relationship which is based on the goitre-induced increase in the secretion of thyrotrophin.

It was for these reasons that the present author decided to study the effect of the thyroid on experimental atherosclerosis. Special attention was paid to the changes in the connective tissue of the vascular wall in atherosclerosis. It was hoped that a study of the so-called tissue factors would throw light also on the effect mechanism of thyroid function in atherogenesis.

The effects of the thyroid on cholesterol and the intermediary metabolism are almost entirely outside the scope of the present work.

THE EFFECT OF THE THYROID ON EXPERIMENTAL ATHEROSCLEROSIS

The first to study induced atherosclerosis in test animals was Anitschkow (1913) who fed cholesterol to rabbits. Dauber and Katz (1942) showed that it is easy to induce vascular atheroma in cockerels by adding small amounts of cholesterol to the diet. Attempts to produce atherosclerosis in cockerels by using other fats instead of cholesterol failed (Peterson *et al.* 1952).

Many investigations have been made into the factors affecting the severity of cholesterol atherosclerosis in animals. The thyroid gland assumed a focal role in these investigations thanks to the old clinical experience that changes in thyroid function have a pronounced effect on the blood cholesterol level in man.

HYPERTHYROIDISM

Several investigators have found that both the thyroid hormone and synthetic thyroid preparations reduce hypercholesterolemia and partially inhibit the development of atherosclerosis in cholesterol-fed rabbits (Friedland 1933; Turner 1933;

Page and Bernhard 1935; Menne *et al.* 1937; von Baló 1939; Boyd 1959). The doses of thyroid used in these experiments were fairly large. According to Breusch and Thiersch (1935), small doses of thyroxin reduce cholesterol-induced atherosclerosis in rabbits.

In the same way as in rabbits, the thyroid hormone reduces the serum cholesterol concentration in cholesterol-fed cockerels and partially inhibits the formation of atherosclerosis (Dauber *et al.* 1949; Stamler *et al.* 1950 c; Stamler 1954). Stamler *et al.* (1950 c) claimed that the effect of thyroid hormone is not based on hypermetabolism for dinitrophenol, which also causes hypermetabolism, has no effect on the atherogenesis of cockerels. They suggested that the thyroid effect is connected with intermediary biochemical reactions. According to Stamler *et al.* (1950 a) the strong lipotropic effect of desiccated thyroid in cholesterol-fed cockerels is based on its stimulating influence on the decomposition of fats. The thyroid-atherosclerosis relationship is probably very complicated, however, in all likelihood involving thyroid effects on protein and vitamin (as well as lipid) metabolism — effects which may influence atherogenesis (Stamler *et al.* 1958 b, c).

Desiccated thyroid also lowers stilbestrol-induced endogenous hypercholesterolemia and diminishes the severity of the atherosclerosis in cockerels (Fleischmann and Fried 1945; Stamler *et al.* 1950 b).

It has also been observed that large doses of thyroxin and thyroid hormone do not essentially reduce cholesterol atherosclerosis (Stamler *et al.* 1958 a) and may even aggravate it (Anitschkow 1933).

The investigations reviewed in the foregoing were concerned with the prophylactic effect of the thyroid, but there are also reports concerning the therapeutic effect of the thyroid on experimental atherosclerosis. Sinitzina (1955) established that thyroid stimulated the resorption of fat from the atheromas after the discontinuance of the cholesterol diet. According to Chakravarti and Mukerji (1956), thyroid hormone likewise accelerates the regression of atheromas in rabbit aorta and coronary arteries. Quite the contrary was noted by Stamler *et al.* (1959) in whose experiment desiccated thyroid retarded the regression of atherosclerotic lesions when the cholesterol diet was stopped.

IODINE

Iodine is closely associated with thyroid function and hence a brief discussion of its effect on experimental atherosclerosis might be in order here.

Most workers have shown that potassium iodine lowers hypercholesterolemia and inhibits the development of atherosclerosis in cholesterol-fed rabbits with an intact thyroid gland (Liebig 1931; Seel and Creuzberg 1931; Turner 1933; Turner and Khayat 1933; Page and Bernhard 1935; Turner and Bidwell 1935). On the other hand, Moses and Longabaugh (1950) reported that potassium iodine had no effect on the atherogenesis of young, growing rabbits. According to some investigations, small doses of potassium iodine can even have an aggravating effect on the cholesterol-induced atherosclerosis in rabbits (Rosenthal 1934; Breusch and Thiersch 1935). Moyer *et al.* (1956) stated that only fairly large doses of potassium iodine have an inhibitory effect on cholesterol-induced atherosclerosis.

In cholesterol-fed cockerels, on the other hand, potassium iodine has not been found to affect hypercholesterolemia or the severity of atherosclerosis (Dauber *et al.* 1949; Stamler *et al.* 1958 a).

Potassium iodine does not stimulate the regression of atheromas in rabbits after discontinuance of the cholesterol diet; on the contrary, the return to normal of the serum cholesterol level is slowed down (Meeker *et al.* 1935; Turner and Bidwell 1937).

Subcutaneously administered iodine has a »thyroxin-like action« in cockerels and rats according to Dvoskin (1947).

HYPOTHYROIDISM

Thyroidectomy has a tendency to aggravate cholesterol atherosclerosis (Shapiro 1927; Turner and Khayat 1933; Menne *et al.* 1937). Thiouracil also increases the serum cholesterol content and aggravates cholesterol-induced atherosclerosis (Steiner and Kendall 1946; Pick *et al.* 1957). Post-thyroidectomy atherosclerosis did not develop in rabbits, at least not during an observation period of 3—4 months, unless a cholesterol diet was fed the animals (Friedland 1933; Turner and Khayat 1933). The literature contains reports of increased atherosclerosis in certain animals following

thyroidectomy (Pineles 1904; Heuper 1944 and 1945; Andrus 1953).

Thyroidectomy, like the thyroid hormone, has no notable effect on the blood cholesterol level in rabbits given ordinary feed (Friedland 1933), but in cholesterol-fed rabbits thyroidectomy raises the cholesterol level considerably (Turner *et al.* 1938). According to the last-mentioned workers, neither hypercholesterolemia nor atherosclerosis developed in some rabbits in spite of a cholesterol-rich diet. Thyroidectomy helped to break the resistance of these animals to atherosclerosis.

Thyroidectomy is incapable of neutralising the inhibitory effect of thyroxin on cholesterol atherosclerosis in rabbits (Turner *et al.* 1938). On the other hand, potassium iodine is incapable of inhibiting hypercholesterolemia in thyroidectomised cholesterol-fed rabbits although the atherosclerotic changes are very small (Turner and Khayat 1933).

Hypothyroidism cancels the protective effect of estrogens against coronary atherosclerosis in cholesterol-fed cockerels (Stamler *et al.* 1956; Pick et al. 1957).

Mention may be made in this connection of some observations on atherosclerosis in man. Heuper (1945) established atherosclerosis on a large scale in connection with cretinism, myxedema and athyreosis in both young and old subjects. According to Wegelin (1925), there is a higher incidence of atherosclerosis in Switzerland in the region of endemic goitre than elsewhere in the country. Nikkilä and Teir (1960) observed that diabetics with an enlarged thyroid showed a lower incidence of coronary sclerosis than diabetics with a small thyroid gland.

THYROTROPHIC HORMONE

Long-term (3—4 months) administration of thyrotrophin alone did not cause atherosclerosis in rabbits, but the cholesterol-thyrotrophin group revealed considerably greater changes than the cholesterol group (Bruger and Fitz 1938). According to Turner and DeLamatar (1942), thyrotrophin reduced the serum cholesterol content whether thyroidectomy had been performed or not. Stamler *et al.* (1958 a) found that thyrotrophic hormone had no effect whatsoever on cholesterol atherosclerosis in cockerels.

SUMMARY

The observations in the literature concerning the effect of thyroid function on experimental atherosclerosis appear to be somewhat conflicting. Generally, hypothyroidism seems to aggravate and hyperthyroidism to inhibit cholesterol atherosclerosis. The effect of thyroxin and thiouracil depends essentially on the size of the dose used and the amount of cholesterol in the diet.

The various animal species differ to some extent. Iodine inhibits atherosclerosis in rabbits, for instance. but has no effect on the cholesterol atherosclerosis of cockerels.

MUCOPOLYSACCHARIDES OF THE VASCULAR WALL AND ATHEROSCLEROSIS

Several theories have been introduced concerning the mechanism of fat deposition in the vascular wall in atherosclerosis. The connective tissue of the vascular wall has been studied in considerable detail especially in the last few years, in an effort to clarify the pathogenesis of atherosclerosis. Advocates of the mucopolysaccharide theory suggest that the acid mucopolysaccharides of the vascular wall have an important role in the genesis of atheroma.

HUMAN ATHEROSCLEROSIS

Virchow propounded the view as long ago as 1856 that the earliest changes in atherosclerosis are the accumulation of a »mucus»-type substance in the intima and the proliferation of connective tissue cells, and that fatty infiltration is only a secondary phenomenon. Similar views were put forward also by Aschoff (1921) in his report on the significance of metachromatic substance in the pathology of atherosclerosis.

An increase in »mucoid» type connective tissue in atherosclerosis was noticed by Björling (1911) and Schultz (1922). Schultz found that fats and calcium had an affinity for tissue of this type. Ssolowjew (1924) and Hueck (1938) reported that mucopolysaccharides were associated with the deposition of cholesterol in the vascular intima.

Both metachromatic substance and mucopolysaccharides were established by Faber (1949) to increase in the vascular wall with age until the 60th year. According to Bunting and Bunting (1953) mucopolysaccharides increased in the media up to the forties but then decreased in consequence of fibrosis. Dyrbye and Kirk (1957) demonstrated that the total amount of mucopolysaccharides in the aorta was clearly diminished in persons aged 60—70 compared with the younger age groups. On the other hand, Miller *et al.* (1952) failed to establish any changes in mucopolysaccharides with age.

No differences in the mucopolysaccharide contents of the different arteries of the same man could be demonstrated by Bassiouni (1955), although the severity of atherosclerosis varied considerably in them. Uotila and his co-workers (1960) used biochemical methods to show that atherosclerosis lowered the acid mucopolysaccharide content of human aorta. They observed moreover that concomitant goitre further intensified the reducing effect of atherosclerosis on aortic mucopolysaccharides *i.e.* the fibrotic and sclerotic effect. Histologically, it is true, an increase in mucopolysaccharides was seen in connection with fresh atherosclerosis changes, but that decreased later owing to subsequent collagenisation. According to Buddecke (1958 a), the atherosclerotic process increased the ground substance of the connective tissue in the vascular wall when its determination was made chemically from amino sugar.

The hypothesis introduced by Faber (1949) was that cholesterol has a tendency to accumulate extracellularly in tissues showing strong metachromasia. He said, further, that irritants and mechanical injuries cause a local increase in mucopolysaccharides in the intima first, intensifying the deposition of fats and thus accelerating the formation of atheroma (tissue factor of atherosclerosis). Several other workers also are of the opinion that tissue damage predisposes to arterial lipid deposition and to atherogenesis (Schlichter *et al.* 1949; Kelly *et al.* 1952; Oester *et al.* 1955; Prior and Hutter 1955). Rinehardt and Greenberg (1949 and 1951) reported that »pyridoxine-deficiency» caused changes in the blood vessels of monkeys reminiscent of human atherosclerosis. A characteristic feature of the lesions was the deposition of a mucoid, metachromatic substance in the intima. Whereas Mann and Andrus (1956) were unable to demonstrate the changes mentioned. Moon

and Rinehart (1952) considered that the accumulation of metachromatic mucopolysaccharides in the intima was primary also in human atherosclerosis, fatty infiltration only secondary. The deposition of »mucinous ground substance» in the intima was regarded by Rinehart (1954) as »the basic lesion of arteriosclerosis». Dock (1946) and Wilens (1951) held that intimal thickening predisposed to the formation of atheroma.

According to Moon (1957), a local degenerative lesion in the internal elastic lamina causes proliferation of mucopolysaccharides and fibroblasts. He regarded this process as the »early nonlipid phase» of atherosclerosis, followed later by a fibrotic scar and degenerative fatty infiltration, and finally by calcification. Several other investigators have held that degenerative changes in the internal elastic lamina are primary factors in the pathogenesis of atherosclerosis (Taylor 1953; Levene 1956; Lindsay and Chaikoff 1957).

Departing from these assumptions, Zugibe and Brown (1958) were unable to establish a correlation between the fat and mucopolysaccharide contents in human aorta. Some investigators have stated that fatty changes are primary alteration in atherosclerosis (Wissler *et al.* 1958; Zugibe and Brown 1960). On the other hand, Noble *et al.* (1957) suggested that human atherosclerosis involved no increase in fat deposition »in early atheromatous areas of the intima». According to Kuntz and Sulkin (1949), the initial atheroma consisted of only a few foam cells in the intima which derive from the endothelium.

EXPERIMENTAL ANIMAL ATHEROSCLEROSIS

The deposition of fat in the intima was regarded as the earliest change in cholesterol-induced atherosclerosis by Buck (1954) and Gore and Barr (1959); mucopolysaccharides increased secondarily. No changes were established by McMillan and Weigensberg (1957) either morphologically or chemically in the aortic mucopolysaccharides of rabbits in cholesterol atherosclerosis.

Some workers, on the other hand, have reported that the increase in mucopolysaccharides was the earliest change in cholesterol atherosclerosis in rabbits (Wang *et al.* 1956; Adlersberg *et al.* 1957).

No intimal proliferation in the initial phase of cholesterol atherosclerosis was observed by Bevans *et al.* (1951 a) in dog, only fat deposition in the media.

It was suggested by Altschuler and Angevine (1951) that the ion exchange reaction between the cations in the blood and partially depolymerised chondroitin sulphates might be of importance in the accumulation of fat and its fixation to the vascular wall. It has in fact been shown that experimentally induced depolymerisation of mucopolysaccharides promotes cholesterol atherosclerosis in rabbits (Benditt *et al.* 1950; Seifter *et al.* 1953; Wang *et al.* 1955 b; Schwartz *et al.* 1958). Depolymerisation was produced in these experiments by intramuscular injection of testicular hyaluronidase.

It has been found that serum mucoproteins are increased in human atherosclerosis (Antonini and Salvini 1957; Schwartz and Gilmore 1958). Elevation of the serum mucoprotein levels in atherosclerosis together with a concomitant alteration in the metachromasia of arterial mucoid substances is interpreted as reflecting an increased depolymerisation of ground substance in this disease (Schwartz and Gilmore 1958). Gore and Barr (1959) were unable to establish changes in serum mucoproteins in cholesterol atherosclerosis in rabbits. According to Hokkanen *et al.* (1960), serum mucoids showed an elevated level in the blood when cockerels were kept on a low-protein-cholesterol diet.

Duff (1935) said that damaged tissue, especially the altered intercellular ground substance in injured areas in the walls of arteries has a marked predilection for the accumulation of lipids. It seems highly probable that some form of injury to the arterial walls is the second essential factor which operates in conjunction with hypercholesterolemia in the production of experimental cholesterol arteriosclerosis in rabbits.

Fat deposition in the intima is the consequence of a state in which the factors causing fat deposition are stronger than the factors which promote their mobilisation and removal (Duff and McMillan 1951).

SUMMARY

Information on the changes in the acid mucopolysaccharides of the aorta in atherosclerosis is somewhat conflicting. Several investigations, however, have established beyond doubt, both chemi-

cally and histologically, that mucopolysaccharides are associated with the genesis of atheromas both in experimental animal atherosclerosis and in human atherogenesis. But there are contradictory opinions as to whether mucopolysaccharide changes are primary or secondary in character. Of especial interest are the observations and hypotheses to the effect that changes do not occur solely in the amounts of mucopolysaccharides but also in their structure and properties.

SULPHUR EXCHANGE OF MUCOPOLYSACCHARIDES

The ground substance of the connective tissue of the vascular wall is composed of mucopolysaccharides which probably originate as the result of the secretion of fibroblasts and small muscle cells (Meyer and Rapport 1951). The acid mucopolysaccharides of the aorta are chiefly chondroitin sulphate B and C (Meyer 1954).

Radio-sulphur injected in sulphate form accumulates in the different organs in the chondroitin sulphates of the connective tissue ground substance (Dziewiatkowski 1951 a, b; Layton 1951 b; Boström 1952; Odeblad and Boström 1952; Davies and Young 1954). The bulk of the radio-sulphur is removed rapidly from the organism (Dziewiatkowski 1945 and 1949). According to Boström (1952), free sulphate disappears almost entirely from the tissues within 48 hours of its injection. Radio-sulphur bound to chondroitin sulphates, on the other hand, is released slowly (Dziewiatkowski *et al.* 1949). The radio-activity of chondroitin sulphates is lowered by a half for 8—16 days, depending on the tissue. This period is termed the biological half-life-time of the sulphur group of chondroitin sulphates (Boström 1952).

S^{35} accumulates profusely in the cardiovascular system and especially in the aorta (Layton 1950; Odeblad and Boström 1952; Boström 1954). Using the quantitative autoradiography technique, it has been shown that the maximum accumulation of S^{35} in the aorta is reached in 48 hours, after which the radioactivity falls slowly and is reduced to half in c. 14 days from the injection (Odeblad and Boström 1953; Boström 1954).

Sulphate sulphur. according to Tarver and Smith (1939), does not participate in the synthesis of sulphur-containing amino acids.

Boström and Åqvist (1952) likewise established that S^{35} injected in sulphate form is not deposited in sulphur-containing amino acids.

The literature on this point can be summarised by saying that the aortic radioactivity c. 2 days after the injection is due in reality to the S^{35} bound to the ester-sulphate group of mucopolysaccharides.

The question of which factors influence the sulphur metabolism of chondroitin sulphates has been studied to some extent. It has been shown that cortisone inhibits the fixation of S^{35} in the tissues, both in vitro (Layton 1951 a; Boström and Jorpes 1954; Boström and Månsson 1954) and in vivo (Layton 1951 a; Boström and Odeblad 1954; Dorfman and Schiller 1958; Kowalewski 1958 a, b; Kowalewski and Strutz 1959). Cortisone is also known to have a general inhibitory effect on connective tissue. But the effect of cortisone on connective tissue, it is worth noting, is contrary to that of thyrotrophic hormone (Iversen 1954).

Hypophysectomy clearly reduces the accumulation of S^{35} in the cartilage of young rats (Ellis *et al.* 1953; Dorfman and Schiller 1958). The growth hormone, again, intensifies the uptake of S^{35} (Dorfman and Schiller 1958). According to Lamberg *et al.* (1956), the accumulation of S^{35} in the retrobulbar connective tissue of guinea pigs increases after injection of thyrotrophin.

A high uptake of S^{35} in several tissues of cock embryos was reported by Layton (1950). The S^{35} uptake decreased considerably, however, when the connective tissue had matured. The uptake remained high even later in the vascular wall. Layton considered this finding to have a significant connection with the tendency of arterial tissue to become sclerosed and calcified.

OBJECT OF THE PRESENT STUDY

As will have appeared from the review of the literature, observations on the effect of thyroid function on atherosclerosis are somewhat contradictory. The same applies to mucopolysaccharide changes in atherosclerosis. Above all, information on the mechanism of action of the thyroid in atherosclerosis is deficient.

This led the author to study the effect of the thyroid on experimental atherosclerosis in cockerels. Special attention was paid to the changes in the connective tissue of the vascular wall in the initial phase of atherosclerosis. The aim was to throw light also on the effect mechanism of the thyroid in atherosclerosis by observing the changes in the mucopolysaccharides of the connective tissue ground substance.

Essentially, the object of the study was to clarify the following points:

1) The effect of thyroxin and thiouracil on cholesterol atherosclerosis and on spontaneous atherosclerosis in cockerels;

2) The effect of thyroxin and thiouracil on the serum cholesterol level in normal and cholesterol-fed cockerels;

3) Do changes occur in the sulphur metabolism of mucopolysaccharides, *i.e.* in the accumulation of radio-sulphur in the aorta, already in the initial phase of atherosclerosis? In other words, is it possible to demonstrate changes in the S^{35} uptake of cholesterol-fed animals in those parts of the aorta where no atherosclerosis has been demonstrable macroscopically so far.

4) Does long-term thyroxin and thiouracil treatment affect the deposition of S^{35} in the aorta of normal and cholesterol-fed cockerels?

5) The distribution of radio-sulphur in normal and atherosclerotic aorta;

6) Connective tissue changes in the aorta in connection with atherogenesis.

MATERIAL

The experimental animals were 5-week-old white Leghorn cockerels fed an ordinary diet before the beginning of the treatment. Cockerels were chosen as test animals because it is easy to induce atheromas in them by means of a cholesterol diet and atherosclerosis in cockerels is the experimentally induced sclerosis in animals that corresponds most closely to human atherosclerosis (Katz and Stamler 1953).

TABLE 1

DIETARY GROUPS AND ORGANISATION OF THE EXPERIMENT

Dietary group	Number of cockerels	Cockerels that died during the experiment	Cockerels sacrificed after 3 months	Cockerels sacrificed after 6 months
I Commercial mash (controls)	27	3	5	19
II » » + cholesterol 1 %	26	1	5	20
III » » + thyroxin 0.00045 %	10	—	—	10
IV » » + thiouracil 0.15 % ..	10	2	—	8
V » » + cholesterol 1 % + thyroxin 0.00045 %	15	3	5	7
VI » » + cholesterol 1 % + thiouracil 0.15 % ..	15	2	5	8
Total	103	11	20	72

The cockerels, 103 in all, were divided into 6 dietary groups for the experiment (Table 1). The basic diet was chicken feed mixture »Kasvatus-Tipu» made by Messrs. Hankkija, containing 4 per cent of crude fat and 20 per cent of crude protein. According to the manufacturer, the mixture contains:

25.0	per	cent	of	maize meal feed
11.3	»	»	»	wheat meal feed
17.0	»	»	»	oatmeal feed
17.4	»	»	»	wheat bran
3.0	»	»	»	coarse soya bean meal
4.0	»	»	»	linseed meal
4.0	»	»	»	sunflower meal
5.0	»	»	»	coarse bone meal
7.0	»	»	»	fish meal feed
4.0	»	»	»	herbage meal
0.35	»	»	»	Hankkija's vitamin A, B_2 and D_3 mixture
1.5	»	»	»	ground limestone for feeding
0.449	»	»	»	common salt
0.001	»	»	»	copper sulphate
100.000	per	cent		

The drugs which were mixed homogeneously with the feed were methylthiouracil (Orion), l-thyroxin (Star) and cholesterol (Merck). The animals were given food and water ad libitum.

Eleven cockerels died during the experiment. The first sacrifice, 5 cockerels from each group except the thyroxin and thiouracil groups, took place after 3 months. Its purpose was to find out whether the effect of thyroxin and thiouracil on cholesterol atherosclerosis changes when the diet is continued over 3 months. The rest of the animals were killed after 6 months. The animals were sacrificed by decapitation.

A test period of this length was chosen to allow any changes that might be caused by the diet to become sufficiently distinct. It was felt advisable, on the other hand, not to use a test period of over 6 months since it is known from the literature that cockerels aged over 6 months begin to show spontaneous atherosclerosis.

The amounts of thiouracil, thyroxin and cholesterol in the feed were relatively small. This was to prevent the drugs from affecting the feed intake and weight development of the cockerels. The thyroxin dosage in particular was small compared with the amounts of thyroxin employed in the experiments reported in the literature.

METHODS

DETERMINATION OF THE SEVERITY OF ATHEROSCLEROSIS

Immediately after the sacrifice, the aorta was freed and with it the iliac and brachiocephalic arteries for a length of c. 1 cm. The aorta was opened longitudinally. The atheromas was measured and plotted on the vascular diagram.

The aortas were classified macroscopically 0—4 by the severity of the atherosclerosis. The classification was performed separately for the elastic aorta (thoracic aorta + brachiocephalic arteries), the muscular aorta (abdominal aorta + iliac arteries) and the aorta as a whole. The evaluation was performed »blindly» in accordance with the following criteria:

Grade 0: No macroscopic changes;
Grade 1: Slight unevenness and yellowness;
Grade 2: Some minor lesions or strong yellowness and unevenness or one major lesion;
Grade 3: Several minor lesions and one major lesion or 2 major lesions;
Grade 4: Two major lesions and numerous other changes or 3 major lesions.

A small elevation clearly demarcated from its environment and measuring 1 × 2 mm at the most was classified as a minor lesion. Major lesions were elongated elevations, over 1 × 2 mm in size, which were clearly demarcated from their environment.

The most important criterion of classification, in accordance with Horlick and Katz (1949) and Katz and Stamler (1953), was the size and number of the atheromas. In one respect, however, the classification differed from that used by these workers: the

degree of atherosclerosis in the aorta was not expressed quantitatively, but the aortas were classified in grades 0—4. The average changes encountered in each grade have been described. The severity of the atherosclerosis in the different groups was expressed as a mean and also in terms of the number of aortas out of the total that were worse than grade 2. Calculating the mean degree of atherosclerosis is mathematically not acceptable since the issue is qualitative and not quantitative calculation. But this procedure was adopted in order to provide a more ready approximate idea of the severity of the atherosclerosis in the different groups.

It was fairly easy in practice to determine the group in which an aorta should be placed. The method obviously gives a fairly reliable picture of the severity of atherosclerosis although the differences between the groups are not equally great.

MEASURING THE S^{35} DEPOSITED IN THE AORTA

Radio-sulphur [1] (as free sulphate in aqueous solution, diluted 0.5 mC/ml) 0.5 mC/kg, was injected into the alar vein 48 hours before sacrifice. A sample of c. 70—80 mg was taken from the region of the aortic arch at a site where no atherosclerotic changes could be seen visually. The organic material in the sample was destroyed according to the method introduced by Boursnell *et al.* (1946), using the wet burning process, and S^{35} was precipitated in $BaSO_4$.

Prepartion of the sample for the measurement of S^{35} uptake:

The sample was weighed immediately on a torsion scale and transferred into a centrifuge tube. Two ml of 10 per cent NaOH was added (keeping quality and digestion). On the following day a mixture containing 9 parts of nitric acid (specific gravity 1.40) and 1 part of concentrated sulphuric acid (specific gravity 1.84) was added to the tube. Approximately 50 mg of copper wire and 4 ml of a mixture containing 3 volumes of nitric acid and 1 volume of 70 per cent perchloric acid were then placed in the tube. The tube was kept for 12 hours at +80°C in a drying cupboard. After this, the contents of the tube were mixed carefully and transferred into a 50 ml Kjeldahl flask. The tubes were rinsed twice with 2 ml of a mixture of nitric acid and perchloric acid. The Kjeldahl bottles were kept in a sand bath

[1] The Radiochemical Centre, Amersham, England.

long enough to evaporate the liquid (c. 5 hours). The bottles were cooled and 5 ml of 15 per cent HCl was added to them, whereupon evaporation was repeated. The bottles were cooled and the precipitate dissolved in 5 ml of 1—N—HCl. The solution was transferred into a 15 ml centrifuge tube. The bottle was washed first with 5 ml and then with 2 ml of 10 per cent $BaCl_2$ solution. The contents of the centrifuge tube were mixed with a glass rod and centrifuged. The precipitate in the centrifuge was washed with alcohol and ether (7—8 ml), followed by centrifugation (3,000 RPM) The precipitate was finally dried in a drying cupboard at +37°C.

Radiosulphur was evenly distributed in the $BaSO_4$ precipitate which was transferred to an aluminium plate for the measuring of radioactivity. This was performed with an end-Mica-window Geiger-Müller counter. The distance from the bottom of the aluminium plate to the window was only c. 3 mm. A total of 1600 impulses was counted in each sample; the standard deviation was 2.5 per cent. Radioactivity was expressed in counts per minute/100 mg of fresh tissue (cpm/100 mg). Radioactivity of the background which varied from 12 to 14 counts per minute was subtracted from the results.

The quantity of precipitate depended on the amount of sulphuric acid used. The precipitate was fairly thick on the aluminium plate (160 mg/sq.cm) and hence the self-absorption of S^{35} was relatively high. In spite of this, the quantity of precipitate was kept so large because small losses occurred in the handling and it was desired to keep the resultant error as small as possible.

AUTORADIOGRAPHY

The distribution of radio-sulphur in the aortic wall was studied by contrast autoradiography, using the stripping film technique. The film used was Kodak Autoradiographic Stripping-Plate AR50, resolution in favourable conditions 15 microns.

The stripping film technique has the advantage that the section under the film can be stained through it if necessary. The autoradiogram and the stained section under it can be viewed concurrently under the microscope. With this technique the autoradiogram and the section are superimposed.

The exposure time was 15 days. Toluidine blue was used for the staining.

Autoradiography technique (Doniach and Pelc 1950; Boyd 1955):

Preparation of the sample:

The piece of tissue was fixed in 80 per cent alcohol and embedded in paraffin wax in the usual manner. A 5 μ thick paraffin section was placed on a glass slide in the usual way. The paraffin was removed with xylol and the glass slide taken through an alcohol series into distilled water. The sample was not allowed to dry, and the slide was kept in distilled water until the section was coated with photographic emulsion.

Covering the section with film emulsion:

The film emulsion on the plate was cut into squares of suitable size in a dark room with a razor blade. The plate was then kept for c. 10 minutes in darkness until the corners of the squares began to work loose from the plate. The pieces of film were then stripped slowly off the glass plate with a razor blade. If it was difficult to detach the emulsion because of excessive moisture the plate was kept for c. 10 minutes in a desiccator. The piece of stripping film was turned and floated emulsion-side downwards on distilled water (21—40°C) where it was allowed to straighten out for 2—3 minutes. The glass slide was entered section-side up under the piece of film floating in the water. The slide was then carefully raised so that the film emulsion covered the section. The film emulsion was allowed to dry at room temperature. Each glass slide was wrapped in black photographic paper. For the duration of the exposure time, 45 days, the samples were kept in a black-walled, dark box at c. +4°C.

Development of the film:

Kodak D—19 b, diluted with distilled water 1: 2, was used as developer. The autoradiogram was kept in the developer for 5 minutes, after which it was washed in distilled water for 30 seconds. The autoradiogram was then transferred in fixing solution for c. 10 minutes (filtered acid-hardener fixer). It was finally washed in distilled water (for c. 30 minutes) and dried at room temperature in a dust-free place. The temperature of the water and the solutions was kept as close to +18°C as possible.

Staining after preparation of the autoradiogram:

The section under the film emulsion was stained by immersing the glass slide for c. 2—3 minutes in 1 per cent toluidine blue solution. The stain was removed from the emulsion in 70 per cent alcohol. Dehydration was done by dipping the glass slide twice in n-butylalcohol. The autoradiogram was finally coated with diatex.

MICRORADIOGRAPHY

The distribution of the mass in the wall of the aorta was examined by ultra soft X-rays. The tube used was the miniature roentgen tube constructed by Engström and Lundberg (1957), and the contact microradiographic technique employed was that introduced by Greulich and Engström (1956).

A c. 3—5 μ thick histological section was fixed on fine-grain photographic emulsion coated with parloidion (Eastman Kodak spectroscopic Plate No. 649). This kept the geometrical inaccuracy as small as possible. The film was exposed in vacuum for c. 15 minutes. The tension of the current in the X-ray tube was 1.5 kV.

Microradiography gives a very accurate picture of biological structures, making it possible to ascertain the dry weight distribution of the histological specimen. The accuracy of the microradiogram with the film emulsion now available is almost equal to that achievable with the light microscope.

Treatment of the sample in ultra-soft microradiography:

A photographic plate was cut with a glazier's knife into pieces of suitable size. The pieces were immersed for 20 seconds in 0.5 per cent parloidion solution (ether and ethanol 1: 1) and then left to dry for a minimum of 30 minutes. The film emulsion thus received a thin layer of parloidion.

A c. 3—5 μ thick paraffin section was floated on the surface of +45°C water until it straightened. The section was then transferred into cold water and put through an alcohol series into absolute alcohol.

The piece of film plate was placed emulsion-side up under the paraffin section floating on the surface of absolute alcohol and the section was fixed to the surface of the parloidion-coated emulsion. This was done by raising the piece of film slowly and simultaneously guiding the section with a brush. The section-emulsion combination was then dried for 5 minutes at room temperature.

The paraffin was removed by immersing the section in pure benzol for 5—10 minutes. In the earlier and also in the following phases of treatment the film was kept horizontal to prevent the section from becoming detached from the surface of the emulsion. To remove any traces of paraffin that might possibly remain in the section, the sample was passed through the following ethanol series: absolute ethanol 60 seconds, 95 per cent ethanol 30 seconds, 80 per cent ethanol 30 seconds, 70 per cent ethanol 30 seconds and 50 per cent ethanol 2 minutes. The section was then returned to absolute ethanol via an alcohol series and kept there for 30 seconds. The emulsion-section combination was then air-dried for 15 minutes after which it was ready for microradiography. The exposure was in vacuum for 15 minutes, voltage of the X-ray tube 1.5 kV. After exposure the section was

removed from the surface of the emulsion by immersing the section-emulsion combination for 3—5 minutes in absolute acetone. Normally the section was freed after some agitation, but sometimes it was necessary to use a brush. Care was taken to ensure that none of the sample remained on the surface of the emulsion. The emulsion was therefore transferred from acetone to 95 per cent ethanol and further for 1 minute to 50 per cent ethanol.

The emulsion was then kept for 5 minutes in undiluted developer (Kodak D-19b). After rapid rinsing in water the emulsion was placed in the acid fixer for 10 minutes. It was finally washed for an hour in filtered water, dried in air and sealed with a cover slip and Canada balsam.

DETERMINATION OF THE SERUM CHOLESTEROL LEVEL

The blood specimens were taken from the alar vein of the cockerels 3 and 6 months after they were placed on the diet. The total serum cholesterol was determined by the Anderson and Keys (1956) procedure of the method of Abell *et al.* (1952).

On the day of sample taking the serum (0.1 ml) as absorbed on Whatman No. 1 filter paper, which was allowed to dry at room temperature. The dried samples were stored in a dark, dry place until analyzed. Hydrolysis of the cholesterol esters was carried out in a thermostat at 70°C for 90 minutes and the cholesterol was extracted with petroleum ether (B.P. 40—60°C) by shaking for 60 seconds three times. The colorimetric reading was made at wavelength 625 mμ with a Beckman B photometer 35 minutes after the addition of Libermann-Burchard reagent. The color reaction occurred in a water bath at 25°C.

Pooled determinations were made by groups after 3 months and individual determinations after 6 months. Three parallel determinations were made for the former and 2 for the latter.

HISTOLOGICAL METHODS

A sample was taken from every thoracic aorta for histological examination. Additional samples were taken from various parts of the aorta as required.

The fresh sample was fixed in 10 per cent neutral formalin for 24 hours. The fixed piece of tissue was dehydrated, cleaned and embedded in paraffin in the usual way. Several sections, c. 3—5 μ thick, were made from each sample and stained by the following methods:

— Toluidine blue staining for metachromasia (Pearse 1960).

Metachromasia is attributable chiefly to the chondroitin sulphates of the ground substance. The stain used was 0.05 per cent aqueous solution (pH 5.0), staining time 16 hours.

— Original periodic-acid-Schiff staining was used as the general connective tissue staining (McManus 1948). Both the ground substance and the elastic fibres are PAS-positive.

— Sudan IV staining (from frozen section) to demonstrate fats (Gatenby and Painter 1946).

— Alcian blue staining to demonstrate mucopolysaccharides (Lison 1954). Mucopolysaccharides stain blue-green and the elastic tissue red with this technique.

— Weigert's resorcin-fuchsin staining for the elastic tissue (Romeis 1948).

The right thyroid gland of each cockerel was taken for histological examination. Several 5 μ sections were made from the centre of the thyroid and stained by Weigert-van Gieson's method (Romeis 1948).

STATISTICAL METHODS

The customary statistical methods were employed in analysing the results. Reference is made to these methods in the standard literature (*e.g.* Hald 1957).

The following terminology was used to denote statistical significance: If the risk level of the test in denoted by p, the effect observed was

almost significant if $P \leq 5$ per cent (*)
significant if $P \leq 1$ per cent (**)
highly significant if $P \leq 0.1$ per cent (***)

The means of the counts for the experimental groups were compared by variance analysis. »Student's» t-test was applied in comparing the group means for the counts with the mean control counts.

Two-way classification was used to study the possible correlation between cholesterol and thyroxin and cholesterol and thiouracil.

The effect of thyroxin and thiouracil on the grade of atherosclerosis was judged by the non-parametric median test (Siegel 1956).

ATHEROSCLEROSIS IN COCKERELS

SPONTANEOUS ATHEROSCLEROSIS

EARLIER INVESTIGATIONS

According to Dauber (1944), spontaneous atherosclerosis begins to appear in cockerels after 6 months of age, and signs of it are demonstrable in c. 50 per cent of cockerels over 1 year old. He found that the spontaneous lesions contained profuse fibrotic tissue. Buddecke (1958 b) showed that the elastic connective tissue in the aorta is reduced and the collagenous connective tissue and structureless ground substance is increased in old hens with spontaneous atherosclerosis.

OWN INVESTIGATIONS

Macroscopic observations: No signs of spontaneous atherosclerosis were observable after a test period of 3 months (Table 2). After 6 months on the other hand, when the cockerels were 7.5 months old, spontaneous atherosclerosis was established in 3 out of 19 cockerels (Table 3). The lesions were exclusively in the region of the muscular aorta. The typical spontaneous lesion was a shallow, pale, elongated intimal thickening. Some lesions, however, were yellow and relatively high, resembling cholesterol-induced atheromas.

Microscopic observations: Spontaneous atheromas displayed profuse fibrotic tissue, scanty foam cells and a little fat. Metachromasia was intensified especially in the fundal parts of the lesions in which Sudan IV staining displayed fatty granules. Alcian blue staining demonstrated a blue-green colour in the lesions caused by the mucopolysaccharides.

Radio-sulphur was deposited more profusely in the spontaneous atheromas than elsewhere in the aorta. The spontaneous lesions

TABLE 2

SEVERITY OF ATHEROSCLEROSIS IN THE DIFFERENT GROUPS AFTER A 3-MONTH DIET

Group	Thoracic aorta						Abdominal aorta						Aorta as a whole					
	Severity						Severity						Severity					
	0	1	2	3	4	Total	0	1	2	3	4	Total	0	1	2	3	4	Total
I Controls	5	—	—	—	—	5	5	—	—	—	—	5	5	—	—	—	—	5
II Cholesterol	—	2	2	—	1	5	—	—	4	—	1	5	—	—	2	2	1	5
III Thyroxin	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
IV Thiouracil	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
V Cholesterol + thyroxin	2	1	1	1	—	5	—	—	2	2	1	5	—	—	2	1	2	5
VI Cholesterol + thiouracil	—	5	—	—	—	5	—	1	3	1	—	5	—	—	3	2	—	5

TABLE 3

SEVERITY OF ATHEROSCLEROSIS IN THE DIFFERENT GROUPS AFTER A 6-MONTH DIET

Group	Thoracic aorta						Abdominal aorta						Aorta as a whole					
	Severity						Severity						Severity					
	0	1	2	3	4	Total	0	1	2	3	4	Total	0	1	2	3	4	Total
I Controls	19	—	—	—	—	19	16	1[1]	1[1]	1[1]	—	19	16	1[1]	1[1]	1[1]	—	19
II Cholesterol	11	6	2	1	—	20	1	—	10	5	4	20	1	—	8	6	5	20
III Thyroxin	10	—	—	—	—	10	6	—	3[1]	1[1]	—	10	6	—	3[1]	1[1]	—	10
IV Thiouracil	7	1[1]	—	—	—	8	4	1[1]	2[1]	1[1]	—	8	4	1[1]	2[1]	1[1]	—	8
V Cholesterol + thyroxin	2	4	1	—	—	7	—	—	1	3	3	7	—	—	1	3	3	7
VI Cholesterol + thiouracil	2	4	2	—	—	8	—	—	5	1	2	8	—	—	3	3	2	8

[1] spontaneous atherosclerosis (arteriosclerosis)

were generally restricted to the intima, but in the vicinity of the major lesions there was narrowing of the media where degeneration of muscle cells was established microscopically.

DISCUSSION

Spontaneous atheromas differed from cholesterol-induced atheromas macroscopically in their shallowness and lighter colour and microscopically in their smaller fat content, smaller number of foam cells and plentiful fibrotic tissue. The histological picture varied considerably and transitional forms were encountered inclusive of lesions reminiscent of cholesterol atheroma.

The present author has followed the terminology of Katz and Stamler (1953) and used the names atherosclerosis for cholesterol-induced aortic changes and spontaneous atherosclerosis for spontaneous lesions. The latter term corresponds to intimal arteriosclerosis employed by some workers.

SUMMARY

In the control group spontaneous atherosclerosis was established in the abdominal aorta of 3 out of 19 cockerels after an experimental period of 6 months. The lesions were characterised by a small fat content and profuse fibrotic tissue.

CHOLESTEROL-INDUCED ATHEROSCLEROSIS

EARLIER INVESTIGATIONS

The investigation by Buck (1954) established that the earliest change in cholesterol atherosclerosis of rabbit is the deposition of foam cells in the intima. Mucopolysaccharides increased secondarily in consequence of the reaction of the connective tissue of the vascular wall to the fatty infiltration. Buck established no correlation between the tissue mucopolysaccharides and the cholesterol invading the tissue. Gore and Barr (1959) said that the early intimal lesions in cholesterol atherosclerosis in rabbits were of purely »fatty character», and they established proliferation of mucopolysaccharides only in connection with older atheromas. They found mucopolysaccharides to be most profuse under the intimal lesions in the media. Bollet *et al.* (1958 and 1960) did not

observe an increase in the mucopolysaccharides of rabbit aorta until after 6 months' cholesterol diet when determined chemically by the orcinol and carbazol methods. Colloidal iron staining, on the other hand, demonstrated an increase in mucopolysaccharides already at an early stage.

Neither morphological nor chemical changes could be demonstrated in the mucopolysaccharides of rabbit aorta in cholesterol atherosclerosis by McMillan *et al.* (1954) and McMillan and Weigensberg (1957). They pointed out, however, that this did not exclude the possibility that changes occur in the ground substance in microscopic dimensions and in the mucopolysaccharides in the degree of polymerisation.

Some workers have held that the proliferation of mucopolysaccharides is the earliest change in cholesterol atherosclerosis in rabbit (Wang *et al.* 1956; Adlersberg *et al.* 1957).

Using autoradiography, Buck (1955) and Forman *et al.* (1960) showed that the deposition of S^{35} is much more profuse in atheromas than in other parts of the rabbit aorta.

OWN INVESTIGATIONS

M a c r o s c o p i c o b s e r v a t i o n s: a 1 per cent cholesterol diet caused pronounced hypercholesterolemia and numerous atherosclerotic changes in the aorta. The severity of the atherosclerosis in the thoracic aorta, the abdominal aorta and the aorta as a whole is shown in Tables 2 and 3 and summarised in Tables 4 and 5.

There were only a few major lesions in the elastic aorta. Atherosclerosis was manifested in this part of the aorta principally as intense yellowness and unevenness and minor lesions. In the thoracic aorta atherosclerosis was considerably scantier than in the abdominal aorta (Table 3) in which large elongated atheromas were established narrowing the lumen of the aorta and also lesions which formed low, transverse ridges in the abdominal aorta. The atheromatous changes were often located at the point where arteries branched off from the aorta and they were especially profuse in the region where the iliac arteries branched off.

Comparison of the 3- and 6-month results shows that the severity of the atherosclerosis showed no appreciable increase when the cholesterol diet continued for over 3 months.

Fig. 1. — Thoracic aorta of the control group. Metachromasia and radioactivity due to S^{35} occur concurrently. (Toluidine blue staining and autoradiogram superimposed, × 10).

Fig. 2. — Cholesterol atheroma of the abdominal aorta with pronounced metachromasia. (Toluidine blue staining, × 10).

Fig. 3. — The abdominal aorta shows a large atheroma and intimal thickening, intensified metachromasia and copious uptake of S^{35}. (Autoradiogram and toluidine blue staining superimposed, × 15).

Fig. 4. — Profuse fat in the intima of the thoracic aorta, but also in the median inner third. (Sudan IV staining, × 10). Toluidine blue staining of the adjacent sections to those in Figs. 5 and 6, autoradiography and toluidine blue staining in Fig. 7 and elastic staining in Fig. 10.

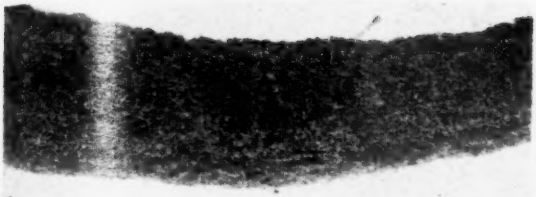
Fig. 5. — Thoracic aorta of the cholesterol group. Intensified metachromasia in the vicinity of the atherosclerotic changes in the subintima and the median inner third. (Toluidine blue staining, × 10).

Fig. 6. — Greater magnification of Fig. 5. Intensified metachromasia in the median inner third. No metachromasia in the intimal foam cells. (Toluidine blue staining, × 120).

Fig. 7. — Combined toluidine blue staining and autoradiography of the thoracic aorta of the cholesterol group. Profuse accumulation of S^{35} in the median inner third, whereas the intimal foam cells show scanty uptake of S^{35}. (× 15).

Fig. 8. — Alcian blue staining of the thoracic aorta which shows atherosclerotic changes in the intima and the median inner third. The blue-green colour caused by metachromasia is intensified in the region of the changes. The intimal foam cells display no blue-green colour. (× 120).

Fig. 9. — Thiouracil-induced lesion in the abdominal aorta. Profuse blue-green colour in the lesion. No blue-green colour elsewhere in the abdominal aorta. (Alcian blue staining, × 15).



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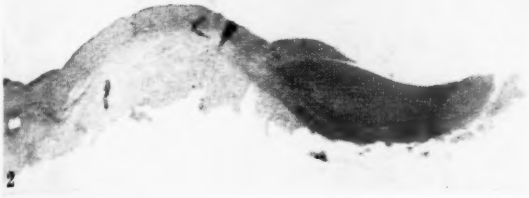

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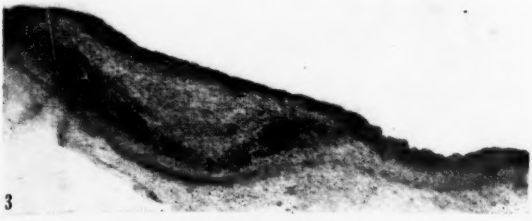

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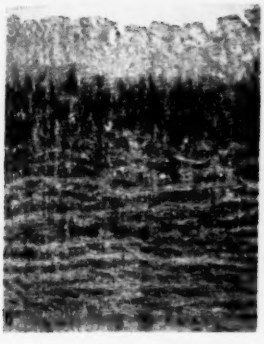

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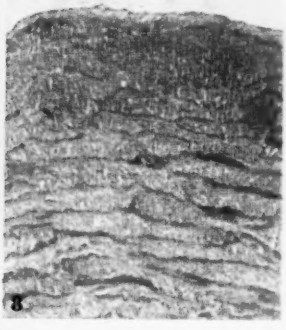

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TABLE 4

AORTIC ATHEROSCLEROSIS AFTER A 3-MONTH CHOLESTEROL DIET

Group	Aortic atherosclerosis				Total serum cholesterol mg% [2]	
	Incidence %	No. of cockerels > Grade 2	No. of cockerels Total	Mean	Mean	± s.e.
Controls	0	0	5	0	103	—
Cholesterol ..	100	3	5	2.8	580	—

TABLE 5

AORTIC ATHEROSCLEROSIS AFTER A 6-MONTH CHOLESTEROL DIET

Group	Aortic atherosclerosis				Total serum cholesterol mg% [2]	
	Incidence %	No. of cockerels > Grade 2	No. of cockerels Total	Mean	Mean	± s.e.
Controls	16	1	19	0.3 [1]	97	5
Cholesterol ..	95	11	20	2.7***	556***	88

[1] = spontaneous atherosclerosis
[2] = pooled sample
s.e. = standard error of the mean
*** = significance level of difference from control group

Microscopic observations:

1. NORMAL AORTA

Toluidine blue staining revealed relatively pronounced metachromasia in the medial and internal third of the normal elastic aorta but none in the region of the muscular aorta. The same was seen in *alcian blue staining* in the blue-green colour caused by the mucopolysaccharides. Both the thoracic and abdominal aorta were *PAS-positive*.

Contrast autoradiography showed a considerably more profuse deposition of S^{35} in the elastic aorta than in the muscular aorta (Figs. 12a and 14b). Radioactivity caused by S^{35} and metachromasia due to mucopolysaccharides occurred concurrently. Both increased in normal thoracic aorta towards the intima (Fig. 1).

Microradiography showed that there was more mass in the elastic fibres than in the ground substance between them.

2. EARLY CHANGES IN CHOLESTEROL ATHEROSCLEROSIS

Accumulation of *fat-containing foam cells* in the intima was a typical feature of the early changes in cholesterol atherosclerosis in cockerels. Proliferation of endothelial cells was observed concurrently. Intensification of *metachromasia* was visible at a fairly early phase, but not prior to the fatty changes.

Early changes in the thoracic and the abdominal aorta differed to some extent. Histologically, atherosclerotic changes were seen fairly early in the subintima and internal third of the media in the thoracic aorta, whereas in the abdominal aorta the changes were limited solely to the intima. The *first microscopically demonstrable change* in the abdominal aorta involved only a few foam cells between the endothelium and the internal elastic lamina.

Sudanophilic materials were found early in the cytoplasm of foam cells and in fibrolasts, but hardly ever in the intercellular space. Free fat was not found except in the deep parts of the large atheromas as the outcome of the breakdown of foam cells.

Intimal foam cells with copious fat in the cytoplasm stained faintly with mucopolysaccharide stainings and showed some radiosulphur accumulation (Figs. 4, 6, 7 and 8).

3. CHANGES WITH THE PROGRESS OF THE ATHEROSCLEROTIC PROCESS

a. *Thoracic aorta.* The severity of the diffuse lesions in the region of the aortic arch increased, but no major lesions proper originated. *Elastic staining* revealed thickening in the vicinity of the changes in the internal elastic lamina and fragmentation in the elastic fibres in the internal third of the media (Fig. 10). The colour caused by the mucopolysaccharides (toluidine blue and alcian blue staining) and the deposition of S^{35} was profuse in the media in the region of the elastic tissue degeneration (Figs. 5, 6, 7 and 8). Fats were always demonstrated in connection with degenerative changes, indicating that the process could be fatty degeneration. Radioactivity was often lowered in the median and outer third of the thoracic aorta near the profuse subintimal changes where there was copious deposition of S^{35} (Fig. 12b).

A few major lesions were found in the descending aorta and close to the boundary of the abdominal aorta the atherosclerotic

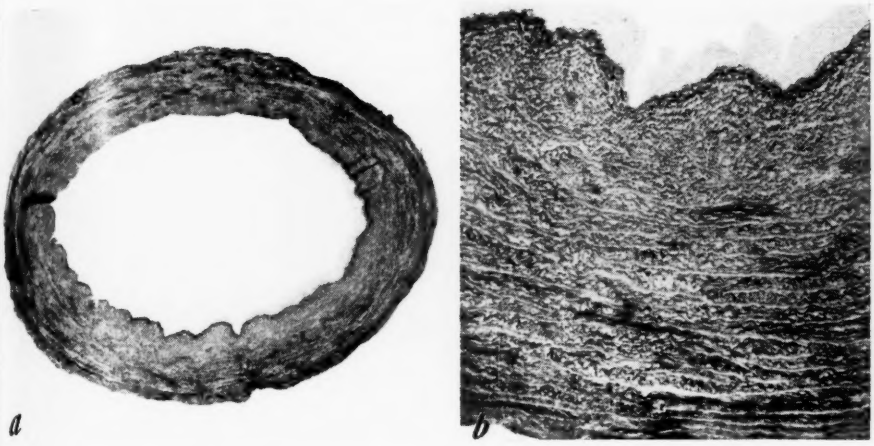

Fig. 10. a. — Thoracic aorta with profuse atherosclerotic changes but no actual atheroma. Thickening and fold formation at the intimal changes in the internal elastic lamina. Degeneration of elastic fibres in the median inner third. (Elastic staining, × 15).

b. Greater magnification of the same site. (× 120). Fig. 4 shows the fat staining and Fig. 5 the toluidine blue staining of the adjacent sections.

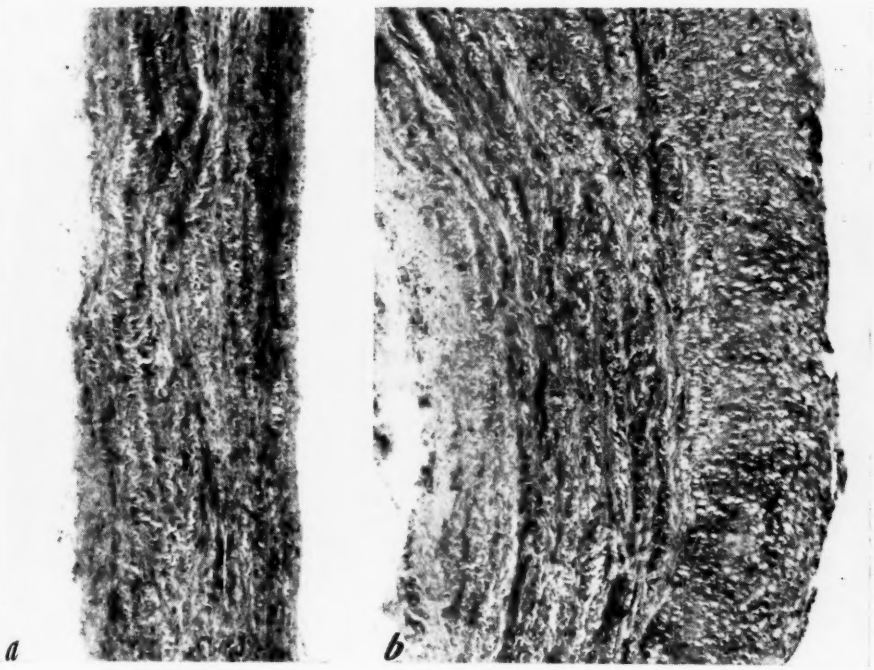

Fig. 11. — a. Descending part of normal thoracic aorta.

b. A major lesion in this part of the aorta. No changes in the media. (Toluidine blue staining, × 10).

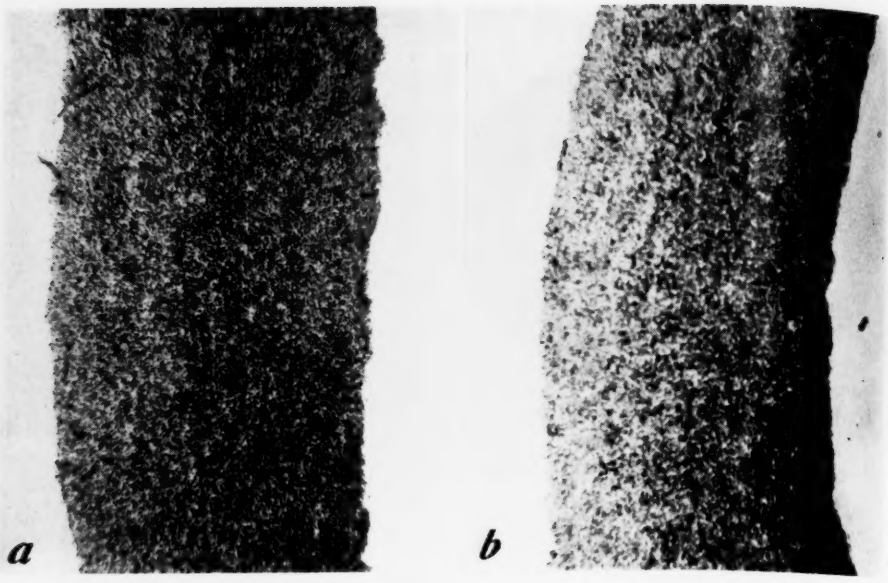

Fig. 12. — a. Autoradiography of the thoracic aorta of a control group cockerel.
b. Aorta of a thiouracil-cholesterol group cockerel. High uptake of S^{35} in the subintima and median inner third. Sudan IV reveals profuse fat at the corresponding site. Radioactivity is lowered in the central and outer third of the media as compared with the control.

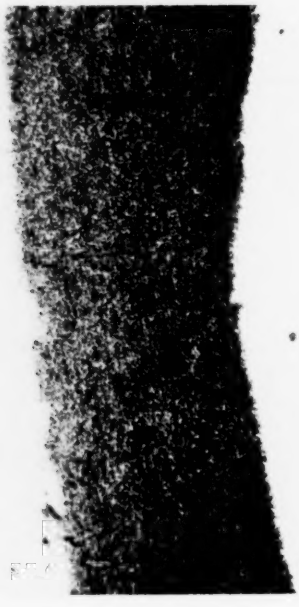

Fig. 13. — Aorta of a thiouracil group cockerel showing a high uptake of S^{35} throughout as compared with the control (Fig. 12 a).

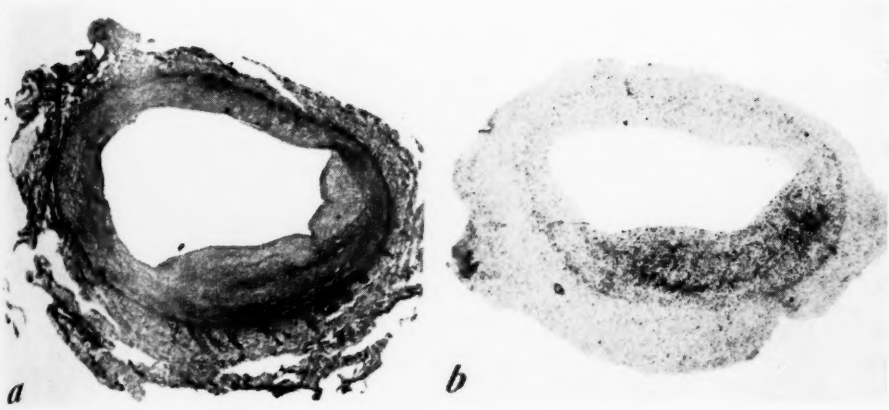

Fig. 14. — a. Large atheroma in the abdominal aorta. (PAS staining, x 15). b. Autoradiography of the same atheroma. Uptake of S^{35} in the atheromatous area high, elsewhere in the abdominal aorta slight. (× 15).

changes were generally bounded by the intima. Fig. 11 shows a major lesion of this type which does not extend to the side of the media.

PAS-positivity also was stronger in the atherosclerotic changes and *microradiography* revealed a decrease in the mass.

b. *Abdominal aorta.* With the advance of the process the abdominal aorta developed major lesions which consisted chiefly of foam cells (Fig. 15). Metachromasia was stronger in fresh atheromas and the colour due to mucopolysaccharides was increased in alcian blue staining (Figs. 2 and 3).

Contrast autoradiography showed that the deposition of S^{35} in the atheromas was considerably more profuse than elsewhere in the aorta (Fig. 14). Especially profuse radioactivity was established on the margins of some atheromas which showed intense metachromasia and in which a zone of fibroblasts and foam cells was seen histologically (Figs. 3 and 17). The uptake of S^{35} was scanty in the central and fundal parts of the large atheromas which displayed profuse free fats and necrosis. In other words, when the atheroma matured the uptake of S^{35} decreased in the same way as metachromasia diminished from the internal parts of the atheroma.

The atherosclerotic lesions in the abdominal aorta were restricted to the intima. This was proved by the finding that the internal elastic lamina was intact even around the large atheromas (Figs. 18).

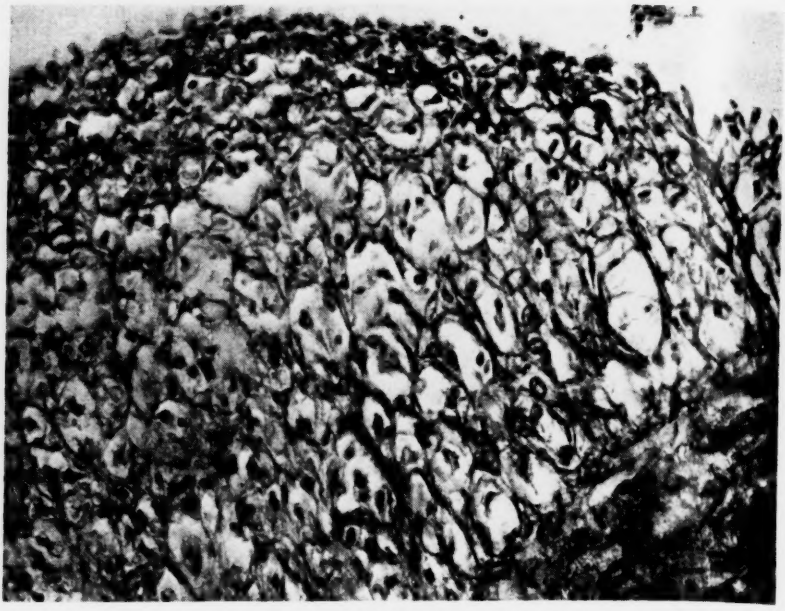

Fig. 15. — A fresh cholesterol atheroma which contains chiefly foam cells. The same atheroma also showed pronounced metachromasia and a high S^{35} uptake. (PAS staining, × 400).

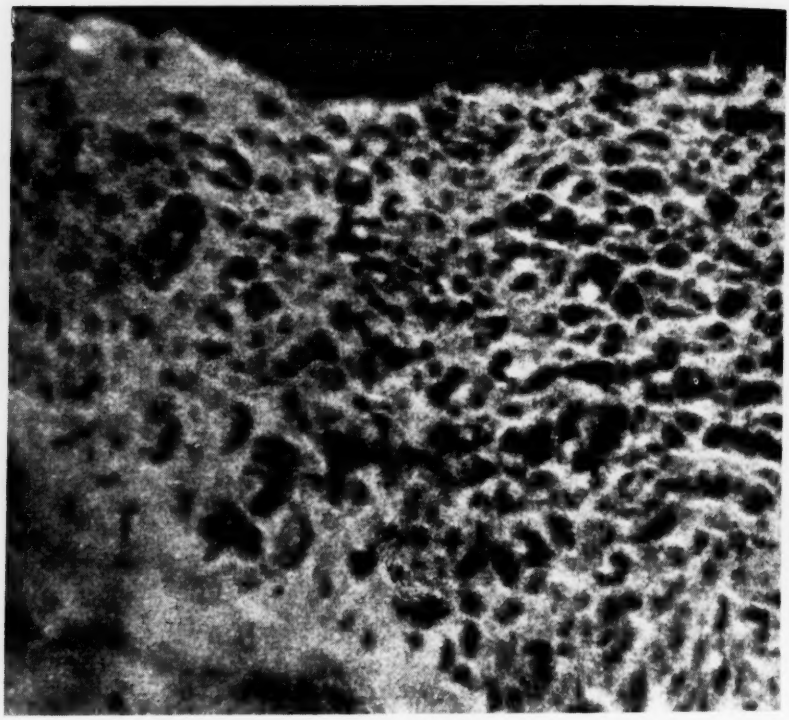

Fig. 16. — Microradiogram of the atheroma in Fig. 15. It shows that the foam cells contain a very small amount of mass (× 400).

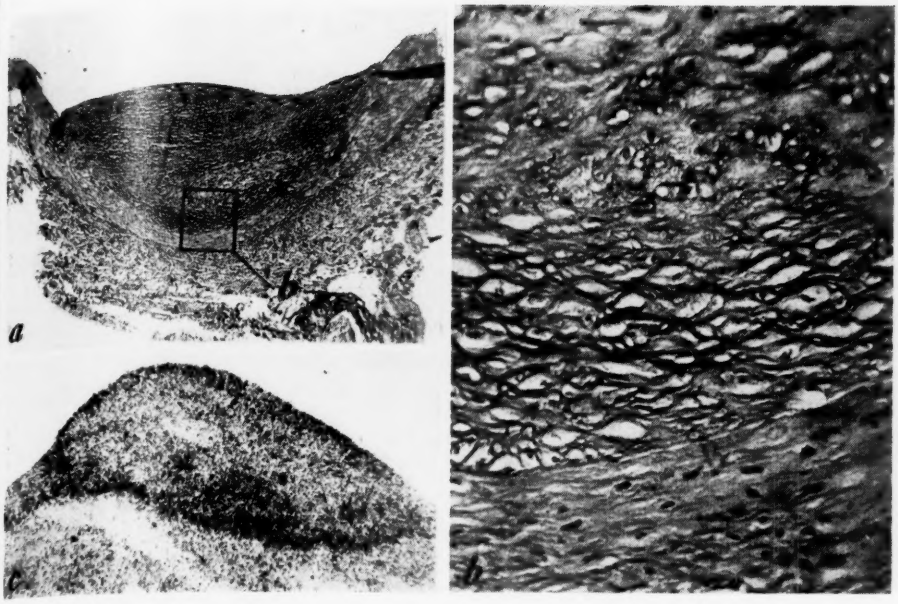

Fig. 17. — a. Cholesterol atheroma in the abdominal aorta. (Toluidine blue staining, × 15).
b. Greater magnification of the above. There was pronounced metachromasia and profuse S^{35} uptake opposite the media in the zone containing fibroblasts and foam cells. Fat staining revealed sudanophilic granules at this site. (Toluidine blue staining, × 400).
c. Autoradiogram of the same atheroma. High S^{35} uptake on the margins of the atheroma.

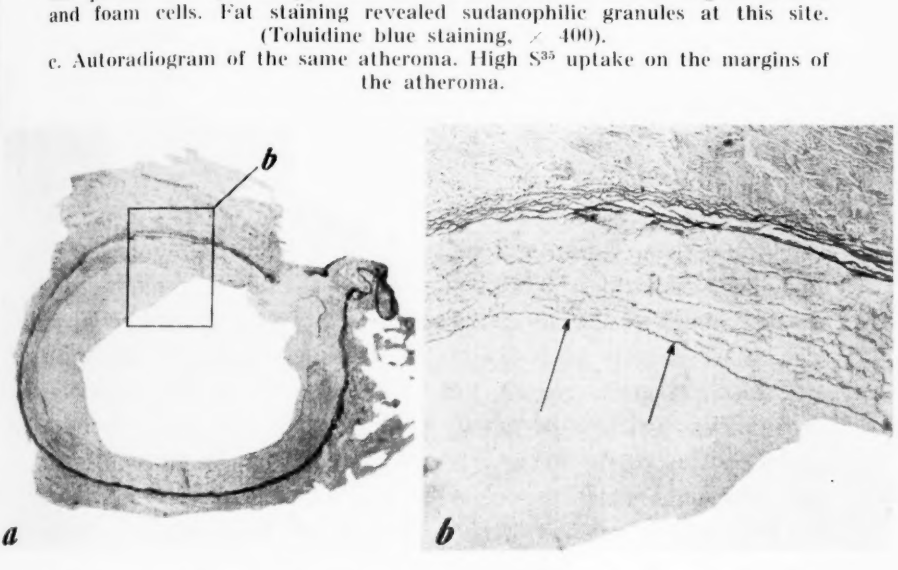

Fig. 18. — a. Large atheroma in the abdominal aorta. Elastic staining. demonstrates an intact internal elastic lamina. (× 15).
b. Greater magnification of the same atheroma. (× 120).

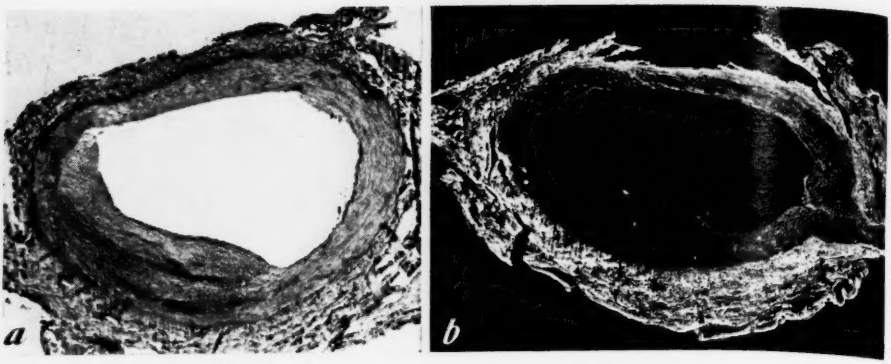

Fig. 19. — a. Atheroma in the abdominal aorta. (PAS staining, × 15) b. Microradiogram of the same atheroma showing a decrease in dry weight in the atheroma and in the vicinity of the atheroma in the media.

The media did show narrowing and, histologically, increased metachromasia. Degeneration of the muscular cells and elastic tissue had occurred at the site and they had been replaced by mucopolysaccharides. Both in the degenerative area in the media and in the atheroma itself the microradiogram showed diminution of mass in comparison with the rest of the media (Fig. 19).

Microradiography revealed a general decrease in the dry weight around the atherosclerotic changes; mass was especially scanty in the foam cells of the atheromas (Fig. 16).

DISCUSSION

The early phase of atherosclerosis is characterised by fatty infiltration of the intima and subintima and by a slight increase in mucopolysaccharides. The last-mentioned finding probably indicates connective tissue reaction, *i.e.* incipient reparation. When the atherosclerotic process advanced the degenerative changes in the elastic tissue increased, but so did the amount of mucopolysaccharides which is obviously to be regarded as an indication of intensified regeneration. The abdominal aorta developed foam cell atheromas which tended to become necrotic as they matured.

The results suggest that fatty changes are primary developments in cholesterol-induced atherosclerosis in cockerels, a process moreover, which is degenerative in character.

SUMMARY

The deposition of fat-containing foam cells in the intima was the earliest change established in cholesterol atherosclerosis in cockerels. Histopathologically, the typical features of cholesterol-induced atherosclerosis were degeneration and fatty infiltration of the intima, the internal elastic lamina and the elastic fibres (in the media of the thoracic aorta), followed by regeneration as the response of the connective tissue and, connected with this, an increase in mucopolysaccharides.

The increase in mucopolysaccharides was confirmed by the following findings made in atherosclerotic lesions: intensified metachromasia and PAS-positivity, intensified mucopolysaccharide colour on alcian blue staining, elevated uptake of S^{35} and reduced dry weight in microradiography.

On the strength of the histological examination, the fatty changes would seem to be primary local changes, the increase in mucopolysaccharides a secondary change only. These observations support the view of the degenerative character of fats in cholesterol-induced atherosclerosis.

THE EFFECT OF THYROXIN ON ATHEROSCLEROSIS IN COCKERELS

SPONTANEOUS ATHEROSCLEROSIS

EARLIER INVESTIGATIONS

Several workers have found that large doses of thyroid hormone cause intimal changes (arteriosclerosis, not atherosclerosis) in animals given ordinary food (Anitschkow 1933; Friedland 1933; von Balò 1939; Heuper 1944 and 1945; Katz and Stamler 1953; Stamler *et al.* 1959). Another important observation, by Stamler *et al.* (1949), was that the thyroid hormone aggravates rather than inhibits the development of spontaneous atherosclerosis in cockerels.

OWN INVESTIGATIONS

Macroscopic observations: A 6-month thyroxin diet caused shallow, pale intimal thickenings in 4 out of 10 cockerels in the abdominal aorta (Table 3, p. 29).

Microscopic observations: The lesions seen visually contained profuse fibrotic tissue and a little fat. They showed increased metachromasia, intensified colour due to mucopolysaccharides and an elevated uptake of S^{35}.

Thyroxin-induced intimal lesions were reminiscent macroscopically and microscopically of spontaneous lesions containing little fat. The thyroxin group displayed slightly more numerous changes than the control group at the end of the 6-month expemental period (Table 6), which would seem to indicate that thyroxin aggravates spontaneous atherosclerosis.

TABLE 6

THE EFFECT OF THYROXIN ON SPONTANEOUS ATHEROSCLEROSIS IN COCKERELS AFTER 6 MONTHS' DIET

Group	Spontaneous aortic atherosclerosis				Total serum cholesterol mg %	
	Incidence %	No. of cockerels > Grade 2	No. of cockerels Total	Mean	Mean	± s.e.
Controls	16	1	19	0.3	97	5
Thyroxin	40	1	10	0.9	108[1]	—

[1] pooled sample
s.e. = standard error of the mean
no significant differences

DISCUSSION

The results support the views of earlier investigators that thyroxin causes intimal thickenings in cockerel aorta and promotes the formation of spontaneous atherosclerosis.

SUMMARY

Small doses of thyroxin caused intimal changes which must be regarded as principally arteriosclerotic. These changes resembled the spontaneous atheromas containing little fat of the control group. Thyroxin seems to aggravate spontaneous atherosclerosis in cockerels.

CHOLESTEROL-INDUCED ATHEROSCLEROSIS

EARLIER INVESTIGATIONS

Turner (1933) conducted the first noteworthy experiments on the effect of the thyroid on experimental atherosclerosis. He found that desiccated thyroid powder (0.4 g/week) clearly reduced cholesterol atherosclerosis but that thyroxin (1.2 mg subcutaneously/week) had only a slight effect on the severity of the disease. Several investigators have since shown that both the thyroid hormone and synthetic thyroid preparations reduce hypercholesterolemia and partially inhibit the development of atherosclerosis in cholesterol-fed rabbits (Friedland 1933; Page and Bernhard 1935; Menne *et al.* 1937; von Balò 1939; Boyd 1959).

In the same way as in rabbits, the thyroid hormone lowers the serum cholesterol concentration in cholesterol-fed cockerels and

partially inhibits the genesis of atherosclerosis (Dauber *et al.* 1949; Stamler *et al.* 1950 c; Stamler 1954). The thyroid doses employed in the experiments reviewed above were relatively large and influenced the animals' intake of food and weight development. According to Breusch and Thiersch (1935), small thyroxin doses (0.05—0.2 mg/day) also reduce cholesterol atherosclerosis in rabbit.

The report published by Stamler *et al.* (1958a) is interesting for the notably different views it contains. According to these authors, large doses of thyroxin (6 mg/day) and of thyroid hormone (1 per cent in food) lower hypercholesterolemia permanently but do not produce an essential reduction of atherosclerosis in the aorta and coronary vessels. As early as 1933 Anitschkow made the observation that large doses of thyroid hormone caused arterial injury and could aggravate cholesterol-induced atherosclerosis in rabbit.

The effect of thyroxin on cholesterol atherosclerosis, Dauber *et al.* (1949) stated, is dependent on the one hand on the thyroxin dose, on the other hand on the amount of cholesterol in the diet.

OWN INVESTIGATIONS

M a c r o s c o p i c o b s e r v a t i o n s: After 3 months on the diet, thyroxin had no essential effect on the severity of cholesterol atherosclerosis (Tables 2 and 7). However, the cholesterol-thyroxin group displayed at this stage slightly more numerous changes than the cholesterol group. At the end of a 6-month diet, thyroxin provoked an increase in the degree of atherosclerosis in the aorta (Tables 3 and 8). It is worth noting that thyroxin nevertheless lowered the serum cholesterol level. The non-parametric median test showed no statistically significant difference in the severity of atherosclerosis between the thyroxin-cholesterol and the cholesterol groups. Macroscopically, however, the difference was distinct and could be classified accordingly. It must be noted that it is difficult to treat qualitative classes statistically — only when there are great differences do significant statistical deviations occur between the groups.

Comparison of the 3-month and 6-month results shows that the atheromatous changes increased slightly when the animals were kept on the cholesterol-thyroxin diet for the longer period.

TABLE 7

THE EFFECT OF THYROXIN ON CHOLESTEROL ATHEROSCLEROSIS IN COCKERELS AFTER 3 MONTHS' DIET

Group	Aortic atherosclerosis				Total serum cholesterol mg%	
	Incidence %	No. of cockerels > Grade 2	No. of cockerels Total	Mean	Mean	± s.e.
Cholesterol ..	100	3	5	2.8	580 [1]	—
Cholesterol-thyroxin ..	100	3	5	3.0	467 [1]	—

TABLE 8

THE EFFECT OF THYROXIN ON CHOLESTEROL ATHEROSCLEROSIS IN COCKERELS AFTER 6 MONTHS' DIET

Group	Aortic atherosclerosis				Total serum cholesterol mg%	
	Incidence %	No. of cockerels > Grade 2	No. of cockerels Total	Mean	Mean	± s.e.
Cholesterol ..	95	11	20	2.7	556	88
Cholesterol-thyroxin ..	100	6	7	3.3	395	58

[1] pooled sample
s.e. = standard error of the mean
no significant differences

Microscopic observations: There was no distinct difference from the findings for cholesterol atherosclerosis.

DISCUSSION

As the average food intake of the cockerels was 135 g/day, the daily thyroxin dose was c. 0.6 mg/animal, *i.e.* only a fifth to a tenth of the doses generally used by the authors cited. The conclusion that thyroxin aggravates cholesterol atherosclerosis differs from the observation by Breusch and Thiersch (1935), viz. that even small doses of thyroxin reduce cholesterol atherosclerosis in rabbit.

The hypothesis introduced by Stamler *et al.* (1958a) was that the influence of thyroid hormone on cholesterol atherosclerosis is the outcome of two opposing processes: »Thyroid-induced partial inhibition of hypercholesterolemia, tending to suppress atherogenesis, and thyroid-induced vascular damage, tending to intensify atherogenesis in animals with preprequisite nutritional metabolic

derangement». This assumption would account for the finding by the present author that small doses of thyroxin intensify atherosclerosis and it explains the observations in the literature to the effect that thyroxin inhibits, has no influence on or even aggravates cholesterol atherosclerosis with the increase in dosage. With small thyroxin doses which did not induce symptoms of hyperthyroidism, the inhibitory effect due to the fall-off in hyperlipemia was not sufficient to nullify the atherosclerosis-intensifying tendency caused by aortic injury. Thyroxin diet alone caused distinct lesions in the abdominal aorta after 6 months in 4 out of 10 cockerels.

SUMMARY

Small doses of thyroxin (0.00045 per cent in food) intensified the severity of atherosclerosis in cholesterol-fed cockerels although they reduced the serum cholesterol concentration. The aggravating effect of thyroxin on atherosclerosis is probably based at least in part on the intimal lesions it causes.

THE EFFECT OF THIOURACIL ON ATHEROSCLEROSIS IN COCKERELS

SPONTANEOUS ATHEROSCLEROSIS

EARLIER INVESTIGATIONS

No report was found in the literature concerning the effect of thiouracil on spontaneous atherosclerosis.

OWN INVESTIGATIONS

Macroscopic observations: A 6-month thiouracil diet caused pale yellow, elongated intimal thickenings (Table 3) in the abdominal aorta of 4 out of 8 cockerels. These thickenings resembled spontaneous atheromas. One of these cockerels also had changes in the thoracic aorta.

The severity of the spontaneous lesions was 0.3 in the control group and 1.0 in the thiouracil group. It seems, thus, that thiouracil intensifies spontaneous atherosclerosis (Table 9) although the non-parametric median test established no statistically significant difference.

TABLE 9

THE EFFECT OF THIOURACIL ON SPONTANEOUS ATHEROSCLEROSIS IN COCKERELS AFTER 6 MONTHS' DIET

Group	Spontaneous aortic atherosclerosis				Total serum cholesterol mg %	
	Incidence %	No. of cockerels > Grade 2	No. of cockerels Total	Mean	Mean	± s.e.
Controls	16	1	19	0.3	97	5
Thiouracil	50	1	8	1.0	129**	6

s.e. = standard error of the mean
** = significance leve | of difference from control group

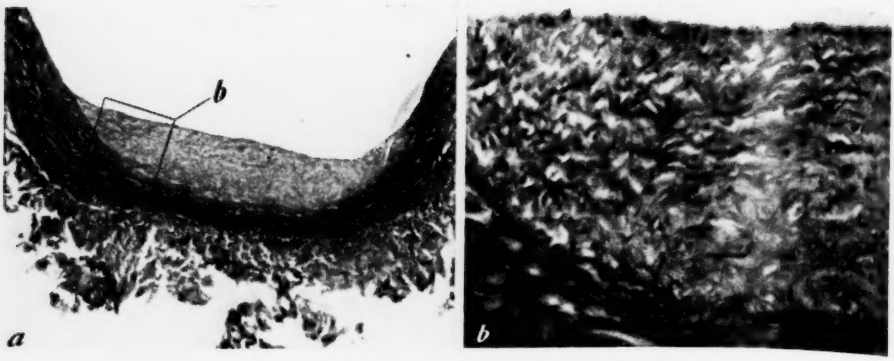

Fig. 20. — a. Typical, shallow thiouracil-induced lesion in the abdominal aorta (PAS staining, × 50).
b. Greater magnification of the above. Profuse fibrotic tissue but no foam cells in the lesion. (× 400).

Microscopic observations: Fig. 20 shows profuse fibrotic tissue in the thiouracil-induced lesion but none of the foam cells which composed the bulk of the fresh cholesterol atheroma (Fig. 15). Sudan IV staining demonstrated fragments of fat in the fundal parts of the lesions (Fig. 21). The same region displayed intensified metachromasia and an elevated S^{35} uptake. Fig. 9 shows alcian blue staining of a thiouracil lesion which reveals the blue-green colour caused by mucopolysaccharides. This colour was not seen elsewhere in the abdominal aorta.

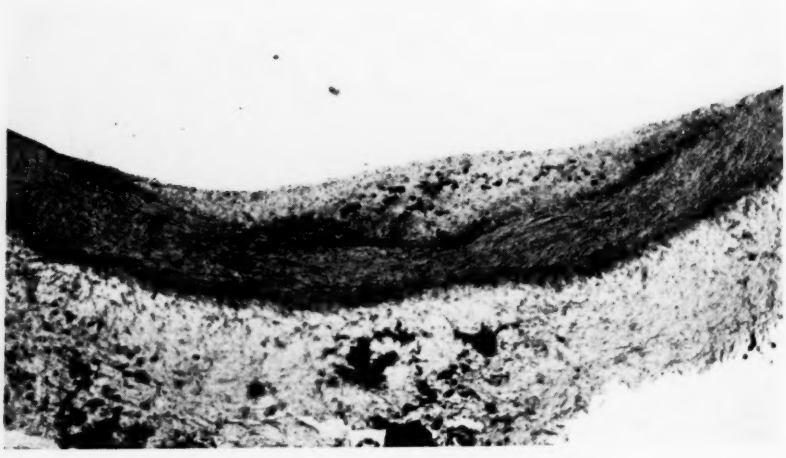

Fig. 21. — Thiouracil-induced lesion in the abdominal aorta. The black spots are fat. (Sudan IV staining, × 50).

DISCUSSION

Thiouracil-induced lesions resembled both microscopically and on gross examination spontaneous atheromas and the intimal changes caused by thyroxin. The lesions of the control, the thyroxin and the thiouracil groups were of the same type in that they were found only in the abdominal aorta (Table 3, p. 29).

Although the histopathological picture of thiouracil-induced lesions varied considerably and some lesions contained foam cells and fat, they differed clearly from cholesterol atheromas. A thiouracil diet alone consequently did not cause atherosclerosis, at least not during the observation period of 6 months, although the serum cholesterol level was considerably elevated ($P. < 0.01$).

SUMMARY

Thiouracil (0.15 per cent in the diet) caused intimal thickenings which resembled spontaneous atheromas. These changes were more numerous in the thiouracil than in the control groups, which suggests that thiouracil aggravates spontaneous atherosclerosis.

Although the serum cholesterol content was elevated, thiouracil alone did not cause actual atherosclerosis, at least not at the end of 6 months' diet, but it produced arteriosclerosis.

CHOLESTEROL-INDUCED ATHEROSCLEROSIS

EARLIER INVESTIGATIONS

According to Pick *et al.* (1957), thiouracil (0.1—0.2 per cent in the diet) considerably raises the serum cholesterol level of cholesterol-fed cockerels, although aortic and coronary sclerosis increase only slightly or at the most moderately. In dog thiouracil intensifies cholesterol atherosclerosis and the degree of hypercholesterolemia is decisive for the development of atheromas (Steiner and Kendall 1946).

Some observations have been made on the effect of hypothyroidism on atherosclerosis and certain hypotheses have been postulated. Uotila *et al.* (1958) said that goitre and arteriosclerosis may have a pathogenetic relationship, possibly through hypothyroidism and the overproduction of thyroid-stimulating hormone by the pituitary gland. Kountz (1950) found that thyroid-

ectomised persons developed in the media degenerative changes revealing plenty of metachromatic substance and the later formation of atherosclerotic plaques. Leary (1936) assumed that human atherosclerosis was caused by a disturbed cholesterol metabolism but that disturbance of the thyroid function was a factor promoting the deposition of cholesterol in the intimal ground substance.

OWN INVESTIGATIONS

Macroscopic observations: Tables 3 (p. 29), 10 and 11 show the effect of thiouracil on cholesterol atherosclerosis. Although the non-parametric median test failed to establish a difference in the severity of atherosclerosis between the cholesterol-thiouracil and the cholesterol groups, the conclusion reached from the macroscopic evaluation was that thiouracil had a slightly intensifying effect on the severity of cholesterol atherosclerosis after the 6-month diet.

Atherosclerosis increased slightly in the cholesterol-thiouracil group when the diet was continued for 6 months.

TABLE 10

THE EFFECT OF THIOURACIL ON CHOLESTEROL ATHEROSCLEROSIS IN COCKERELS AFTER 3 MONTHS' DIET

Group	Aortic atherosclerosis				Total serum cholesterol mg%	
	Incidence %	No. of cockerels > Grade 2	No. of cockerels Total	Mean	Mean	± s.e.
Cholesterol ..	100	3	5	2.8	580 [1]	—
Cholesterol-thiouracil ..	100	2	5	2.4	1690 [1]	—

TABLE 11

THE EFFECT OF THIOURACIL ON CHOLESTEROL ATHEROSCLEROSIS IN COCKERELS AFTER 6 MONTHS' DIET

Group	Aortic atherosclerosis				Total serum cholesterol mg%	
	Incidence %	No. of. cockerels > Grade 2	No. of. cockerels Total	Mean	Mean	± s.e.
Cholesterol ..	95	11	20	2.7	556	88
Cholesterol-thiouracil ..	100	5	8	2.9	1067*	224

[1] pooled sample
s.e. = standard error of the mean
* significance level of difference from cholesterol group

Microscopic observations: The findings were similar to those established for the cholesterol group.

DISCUSSION

The result obtained, that thiouracil elevates sharply the serum cholesterol level but intensifies the severity of atherosclerosis in cholesterol-fed cockerels only slightly, agrees with the observations made by Pick *et al.* (1957).

Thiouracil exerts an aggravating effect on cholesterol atherosclerosis by raising the serum cholesterol level. Also aggravating in their effect, probably, are the thiouracil-induced intimal lesions which were present in 4 out of 8 cockerels in the group given thiouracil alone.

Thiouracil is not known to have a direct effect on connective tissue. On the other hand, it inhibits the synthesis of the thyroid hormone and thus leads to an increased production of thyrotrophin. Thyrotrophin has a direct stimulating effect on connective tissue, which results in an increase in mucopolysaccharides in the connective tissue (Asboe-Hansen and Iversen 1951; Asboe-Hansen *et al.* 1952). It was mentioned in connection with the review of the literature that several investigators regard an increase in the ground substance of the vascular wall as a factor promoting atherosclerosis.

The effect mechanism of thiouracil must probably involve additional factors which partially offset the influence of the above-mentioned aggravating factors for the degree of cholesterol atherosclerosis did not increase as much as these factors suggest it should have.

SUMMARY

Thiouracil (0.15 per cent in the diet) raised the serum cholesterol concentration sharply in cholesterol-fed cockerels, but the severity of atherosclerosis increased only slightly. Thiouracil has an aggravating effect on cholesterol atherosclerosis since it elevates the serum cholesterol level, probably also because it causes intimal lesions and possibly by causing increased production of thyrotrophin. The effect mechanism of thiouracil obviously involves additional factors which partially neutralise the aggravating factors mentioned.

SERUM CHOLESTEROL CONCENTRATION AND THE SEVERITY OF ATHEROSCLEROSIS

EARLIER INVESTIGATIONS

It has been shown by Horlick and Katz (1949) and Bevans *et al.* (1951 a, b) that there is something approaching a direct correlation between the degree and duration of hyperlipemia, on the one hand, and the severity of cholesterol-induced atherosclerosis, on the other hand.

The feeding of rabbits and cockerels with cholesterol causes hypercholesterolemia in which the ratio total cholesterol/phospholipids (C/P) is elevated in the plasma (Hirsch and Weinhouse 1943; Stamler *et al.* 1950 d). It has been suggested that it is just this increase in the C/P ratio that is decisive for the genesis of atherosclerosis and that the relative increase in phospholipids in the serum, *i.e.* a lowered C/P ratio, inhibits cholesterol atherosclerosis (Ladd *et al.* 1949; Payne and Duff 1950; Orma 1957). According to Katz and Stamler (1953), there is no correlation between the plasma C/P ratio and atherosclerosis and cholesterol levels per se are more significant for cholesterol-induced aorta atherosclerosis than C/P ratios.

OWN INVESTIGATIONS

The degree of atherosclerosis in the different groups and the total serum cholesterol level are summarised in Tables 12 and 13.

In cockerels given normal food, thiouracil elevated the serum cholesterol level to some extent but thyroxin had no essential effect on the cholesterol content.

Small doses of thyroxin seemed to aggravate the atherosclerosis somewhat, although the serum cholesterol level fell. Despite the fact that thiouracil raised the serum cholesterol level the cholesterol atherosclerosis was intensified only relatively slightly.

TABLE 12

AORTIC ATHEROSCLEROSIS AND SERUM CHOLESTEROL LEVEL AFTER 3 MONTH'S DIET

Group	Aortic atherosclerosis			Total serum cholesterol mg% [2]
	No. of cockerels		Mean	
	> Grade 2	Total		
I Controls	0	5	0	103
II Cholesterol	3	5	2.8**	580
III Thyroxin	—	—	—	—
IV Thiouracil	—	—	—	—
V Cholesterol + thyroxin	3	5	3.0**	467
VI Cholesterol + thiouracil	2	5	2.4**	1690

TABLE 13

AORTIC ATHEROSCLEROSIS AND SERUM CHOLESTEROL LEVEL AFTER 6 MONTH'S DIET

Group	Aortic atherosclerosis			Total serum cholesterol mg%	
	No. of cockerels		Mean		
	> Grade 2	Total		Mean	± s.e.
I Controls	1	19	0.3 [1]	97	5
II Cholesterol	11	20	2.7***	556***	88
III Thyroxin	1	10	0.9 [1]	108 [2]	—
IV Thiouracil	1	8	1.0 [1]	129**	6
V Cholesterol + thyroxin	6	7	3.3**	395**	58
VI Cholesterol + thiouracil....	5	8	2.9***	1067**	224

[1] spontaneous atherosclerosis (arteriosclerosis)
[2] pooled sample
s.e. = standard error of the mean
** } significance level of difference from control group
*** }

The non-parametric median test failed to establish statistical differences between the cholesterol, cholesterol-thyroxin and cholesterol-thiouracil groups in the severity of atherosclerosis. But differences were demonstrable in macroscopic classification. Even if the atherosclerosis were assumed to be of roughly the same grade in these three groups, there is a distinct disproportion between the serum cholesterol concentration and the severity of

the atherosclerosis. In other words, the ingestion of thyroxin and thiouracil disperses the positive correlation between the serum cholesterol content and the grade of aorta atherosclerosis.

DISCUSSION

Stamler *et al.* (1950 c) reported that thyroid hormone increased phospholipids and lowered the C/P ratio in cholesterol-fed rabbits. Thyroxin thus affects the serum cholesterol level and the lipid components by inhibiting the genesis of atherosclerosis, and it is consequently impossible through this mechanism to account for the disproportion established in the present series between the serum cholesterol level and atherosclerosis.

Several workers have come to the conclusion that in addition to the regulation of the serum cholesterol concentration, the effect of the thyroid on atherosclerosis is based on its effect on the tissues of the fat deposition mechanism (Page and Bernhard 1935; Heuper 1944 and 1945; Katz and Dauber 1945; Gubner and Ungeleider 1949; Stamler *et al.* 1950 a, b). The permeability of tissues and especially of the endothelium is of great significance in atherogenesis (Kellner and Dju Chang 1951; Adlersberg *et al.* 1954; Wang *et al.* 1955 a). Some investigators, in fact, hold that the effect of thyroxin is based on the changing of the permeability of the vascular wall (Davson and Danielli 1943; Gubner and Ungeleider 1949). According to Lange (1944), thyroid hormone and iodides which inhibit cholesterol-induced atherosclerosis lower the permeability of the intima, whereas permeability increases in myxedema. Mardones and Monsalve (1951) assume that the thyroid hormone affects atherosclerosis by inducing a rise in cellular cytochrome c.

Especial significance attaches to the finding that the severity of atherosclerosis was somewhat greater in the cholesterol-thyroxin group than in the thiouracil-cholesterol group although the serum cholesterol content of the former was only about one-third of that of the latter group. It can be concluded from this that the essential influence of thyroid function on cholesterol atherosclerosis is based on a mechanism other than regulation of the blood cholesterol level.

SUMMARY

The ingestion of thyroxin and thiouracil disperses the positive correlation between the serum cholesterol content and the severity of cholesterol-atherosclerosis. It seems obvious from the observation made that the most essential effect of thyroid function on cholesterol-induced atherosclerosis in cockerels is based on a mechanism other than the changing of the serum cholesterol level.

THE EFFECT OF CHOLESTEROL, THYROXIN AND THIOURACIL ON THE SULPHUR EXCHANGE OF AORTIC MUCOPOLYSACCHARIDES

EARLIER INVESTIGATIONS

The deposition of S^{35} in the aorta was found by Buck and Heagy (1958) and Forman *et al.* (1960) in rabbits and by Kowalewski (1959) in cockerels to increase considerably after a long-term cholesterol diet. Buck (1955) and Forman *et al.* (1960) showed by autoradiography that the increase in the radioactivity of the aorta is due to the liberal accumulation of S^{35} in the atheromas. It is noteworthy that radioactivity is not increased in certain other organs of cholesterol-fed animals although ample cholesterol penetrates those organs (Buck and Heagy 1958; Kowalewski 1959).

The accumulation of S^{35} in the cartilage was lowered in young rats given thiouracil for 2—3 days. Thyroxin had an inhibitory effect or no effect at all depending on the dose (Dziewiatkowski 1951 c). The same author stated that the inhibitory effect of thyroxin on S^{35} uptake was based on its ability to accelerate the maturation of the cartilage.

According to Boström and Jorpes (1954) thiourea and semicarbazol inhibit the deposition of S^{35} in cartilage.

OWN INVESTIGATIONS

Table 14 shows the severity of the atherosclerosis and accumulation of S^{35} in the aorta by groups and as means.

C h o l e s t e r o l: The deposition of S^{35} in the aorta was significantly increased ($P < 0.01$) in the cholesterol group in comparison with the controls. As the samples were taken from a site with no

TABLE 14

DEPOSITION OF RADIO-SULPHUR IN THE AORTA IN THE DIFFERENT GROUPS AFTER A 6-MONTH DIET

Group	N	S^{35} uptake in the aorta cpm/100 mg of fresh tissue				Severity of aortic atherosclerosis Mean
		Range	Mean	± s.e.	P	
I Controls	19	85—230	156	8		0.3[1]
II Cholesterol ..	20	137—213	188	8	<0.01	2.7
III Thyroxin	10	145—231	176	12	>0.05	0.9[1]
IV Thiouracil ..	8	174—236	201	13	<0.01	1.0[1]
V Cholesterol + thyroxin	7	196—305	263	14	<0.001	3.3
VI Cholesterol + thiouracil	8	169—301	247	13	<0.001	2.9

P = significance level of difference from control group
s.e. standard error of the mean
[1] = spontaneous atherosclerosis (arteriosclerosis)

macroscopically observable atherosclerotic changes, it can be concluded that changes in the sulphur metabolism of mucopolysaccharides occur already in the so-called early phase of atherosclerosis.

Thyroxin: The radioactivity of the aorta was slightly greater in the thyroxin group than in the controls. Thyroxin thus had a slight uptake-increasing influence, but the difference was not statistically significant.

The maximum radioactivity was measured in the cholesterol-thyroxin group ($P < 0.001$). Thyroxin thus intensifies the increase in S^{35} uptake induced by cholesterol in the aorta. Two-way classification established interaction between thyroxin and cholesterol ($P < 0.01$).

Thiouracil: Thiouracil increased significantly the uptake of S^{35} in the aorta ($P < 0.01$). Fig. 13 is an autoradiogram of the aorta of a thiouracil group cockerel which reveals a generous uptake of S^{35} in the aorta (the aorta of the control group is seen in Fig. 12 a); radio-sulphur was distributed normally in the wall of the aorta.

High radioactivity was measured in the cholesterol-thiouracil group ($P < 0.001$). Thiouracil thus enhanced the increase in S^{35} up-

take induced by cholesterol. Two-way classification established no interaction between thiouracil and cholesterol; the effect in question was one of summation.

DISCUSSION

Both cholesterol and the disturbance of thyroid function had a distinct effect on the deposition of S^{35} in the chondroitin sulphates of the aorta. The finding that both thyroxin and thiouracil enhanced the elevated S^{35} uptake induced by cholesterol is not necessarily conflicting. The uptake of S^{35} is governed by several different factors such as the amount and metabolism and obviously also the changes in the structure of mucopolysaccharides. Metachromasia and radioactivity caused by S^{35} occurred concurrently in the aorta, and it is known that the intensity of metachromasia depends on the quantity of mucopolysaccharides and also on their degree of polymerisation and sulphation (McManus 1954).

The conclusion arrived at by Dzietwiatkowski (1951 c), that thiouracil inhibits the uptake of S^{35} in the mucopolysaccharides of cartilage, is not comparable with the results of the present experiment as the former was concerned with the short-term effect of thiouracil.

According to Schwartz *et al.* (1958) cholesterol feeding of rabbits causes hypercholesterolemia and changes in the connective tissue ground substance of the vascular wall. They held that cholesterol feeding is merely one of many ways in which tissue damage may be induced and that the latter is of greater importance in atherogenesis. The results obtained in the present series support this view. An increase in S^{35} uptake was established in the cholesterol group also in places in the aorta where no atherosclerotic changes could be seen on gross examination. That the uptake of S^{35} was not proportional to the severity of the aorta atherosclerosis in question is further support for the theory that the increased uptake in the cholesterol group is not attributable to intensified atherosclerosis but to the effect exerted by cholesterol on the mucopolysaccharides of the ground substance. It can probably be assumed that changes occur in the early phase of atherosclerosis in the metabolism and structure rather than in the amount of the mucopolysaccharides. It is possible that these changes in the properties, metabolism and

structure of mucopolysaccharides are primary phenomena in experimental atherosclerosis in cockerels and not fatty changes as might have been expected from the histological examination.

SUMMARY

Cholesterol increased significantly the deposition of S^{35} at a site in the aorta where no atheromatous changes were discernible macroscopically. In other words, changes occurred in the early phase of atherosclerosis in the metabolism, and probably also structure, of the mucopolysaccharides. The changes in the ground substance are possibly primary in cholesterol atherosclerosis, and the amount of the mucopolysaccharides does not increase until later.

Thiouracil increased significantly the uptake of S^{35} in the aorta. Both thyroxin and thiouracil enhanced the elevated uptake of S^{35} induced by cholesterol. Hence, disturbed thyroid function altered the metabolism of the aortic mucopolysaccharides. It is probable that these changes in the metabolism of the aortic wall are of significance in the genesis of cholesterol atherosclerosis.

THE EFFECT OF CHOLESTEROL, THYROXIN AND THIOURACIL ON THE THYROID GLAND OF COCKERELS

EARLIER INVESTIGATIONS

It was reported by Kipshidze (1957) that the accumulation of J^{131} in the thyroid gland was reduced in rabbits given cholesterol for 6 months and that this indicated a weakening activity of the thyroid. Prostjakov *et al.* (1957) proved by means of radioactive iodine that thyroid function was lowered in man suffering from severe atherosclerosis. Orma, too (1957), said that cholesterol-feeding tended to inactivate the thyroid of cockerels. Marx *et al.* (1948) found that a diet rich in cholesterol prolonged significantly the life of rats given toxic doses of thyroid hormone. According to Saegesser (1933), too, the effects of thyroid hormone could be inhibited by feeding the animals cholesterol. It was found by Winebrenner and Marx (1949) that cholesterol diminished the basal metabolism of animals given thyroxin, but not of normal animals.

OWN INVESTIGATIONS

The mean thyroid weights of the different groups are given in Table 15.

C h o l e s t e r o l: After three months' diet the thyroid was heavier in the cholesterol-fed group than in the control group, but the difference was not statistically significant. Six months' cholesterol diet increased the thyroid weight almost significantly ($P < 0.05$). Histological examination showed an increase in the relative colloid content of the thyroid in the cholesterol group in comparison with the controls (Fig. 22).

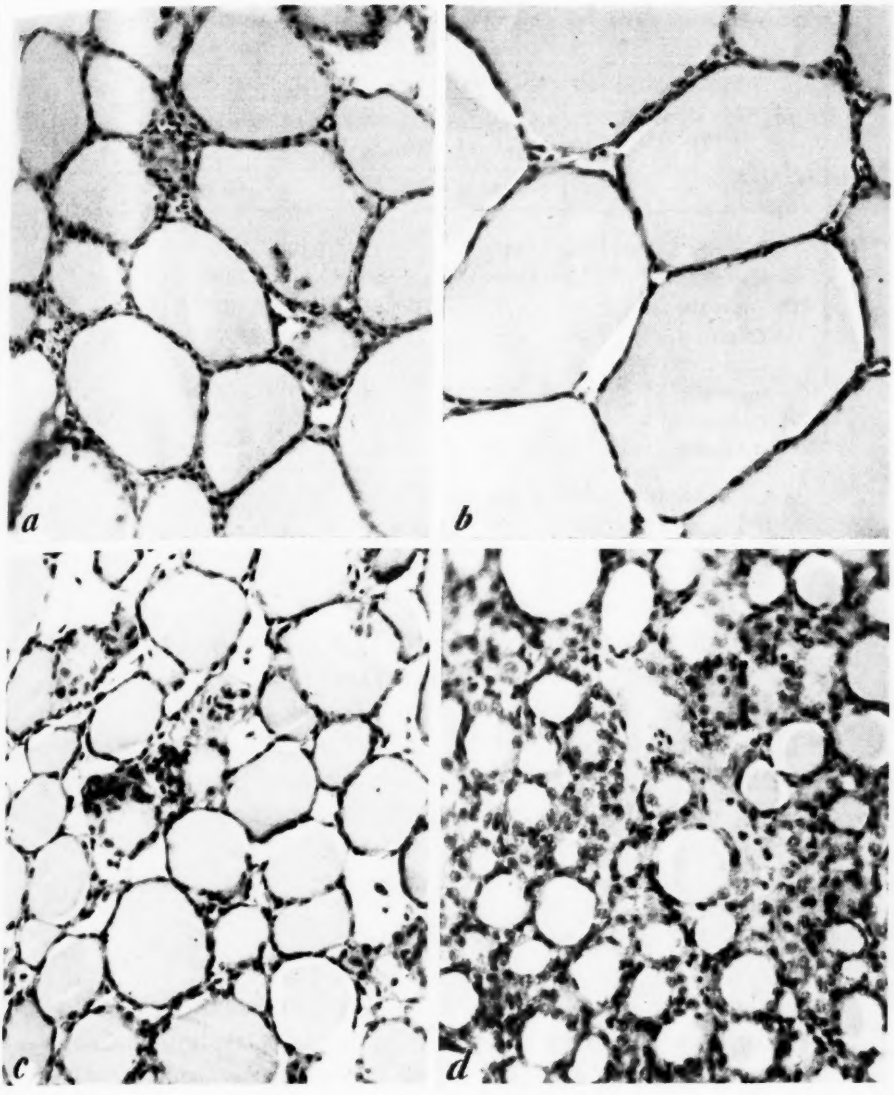

Fig. 22. — Histological picture of cockerel thyroid after 6 months' diet. a. *Control.* b. *Cholesterol:* relative colloid content increased, thyroid inactivated. c. *Thyroxin:* follicles small, thyroid inactivated. d. *Thiouracil:* relative epithelial content sharply increased, thyroid activated.
(Weigert-van Gieson, × 400)

TABLE 15

WEIGHT OF THE THYROIDS IN THE DIFFERENT GROUPS AFTER 3 MONTHS' AND 6 MONTHS' DIET

Group	3 months			6 Months		
	N	Weight of thyroids g		N	Weight of thyroids g	
		Mean	± s.e.		Mean	± s.e.
I Controls	5	148	7	19	201	14
II Cholesterol	5	199	30	20	245*	12
III Thyroxin	—	—	—	8	62***	4
IV Thiouracil	—	—	—	8	1878*	586
V Cholesterol + thyroxin	5	33***	5	7	51***	9
VI Cholesterol + thiouracil.....	5	991**	247	10	1237***	235

s.e. = standard error of the mean

*, **, *** significance level of difference from control group

T h y r o x i n: Pronounced atrophy ($P < 0.001$) of the thyroid was established in both groups given thyroxin. Microscopy revealed that the follicles were small and the epithelium low (Fig. 22 c), and hence the histological picture also indicates atrophy.

T h i o u r a c i l: The thyroid grew considerably in all the groups of cockerels fed with thiouracil. The proportion of epithelium was histologically sharply increased (Fig. 22 d).

DISCUSSION

A decrease in the relative colloid content signifies activation of the thyroid, an increase signifies its inactivation (Lamberg 1953; Wahlberg 1955). The increase in the relative colloid content in the cholesterol group indicates that cholesterol feeding tends to inactivate the thyroid. The result concurs with the observations reviewed above made by several authors. As, in addition, the weight of the thyroid grew in the cholesterol group, it may perhaps be said that a cholesterol diet caused a mild colloid goitre in cockerels.

SUMMARY

Cholesterol-feeding caused enlargement of the thyroid and a tendency to inactivate the thyroid. Thyroxin caused pronounced atrophy of the thyroid. Thiouracil increased considerably the weight and relative epithelial content of the thyroid.

THE EFFECT OF DIFFERENT DIETS ON THE WEIGHT OF COCKERELS

The weights of the individual cockerels were much the same at the beginning of the experiment. During the experiment they were weighed once a month. The weight development in the different groups is shown in Fig. 23. Table 16 shows the weight means by

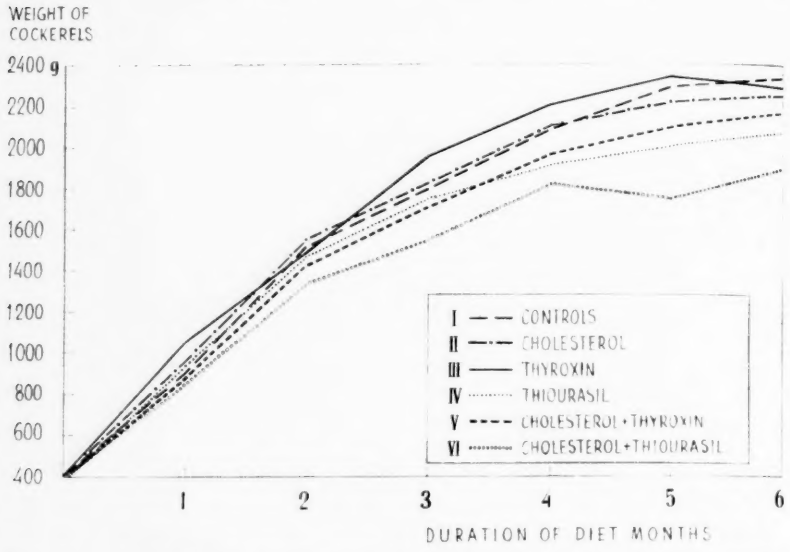

Fig. 23. — The weight development of the animals in the different groups.

groups after 3 months' and 6 months' diet. The weight of the animals began to fall after 3—4 months of diet in both the cholesterol-thiouracil and the simple thiouracil group. By the end of the experiment the weight had declined significantly ($P < 0.001$) in the former and almost significantly ($P < 0.05$) in the latter group compared with the controls. The cholesterol-thiouracil group had a slightly reduced food intake.

TABLE 16

THE WEIGHT OF THE COCKERELS IN THE DIFFERENT GROUPS AFTER 3 AND 6 MONTHS' DIET

Group	3 months			6 months		
	No. of cockerels	Weight g		No. of cockerels	Weight g	
		Mean	± s.e.		Mean	± s.e.
I Controls ..	5	1820	98	19	2284	49
II Cholesterol	5	2076	58	20	2222	68
III Thyroxin ..	—	—	—	8	2228	34
IV Thiouracil ..	—	—	—	8	2080*	81
V Cholesterol + thyroxin	5	1950	57	7	2184	84
VI Cholesterol + thiouracil	5	1940	72	10	1906***	64

s.e. = standard error of the mean

*, **, *** significance level of difference from control group

There was no essential difference between the other groups either in food intake or in weight development.

SUMMARY

Both the thiouracil and the thiouracil-cholesterol diet caused a weight loss compared with the controls. This, however, is probably of no significance in the evaluation of the results of the experiment.

GENERAL DISCUSSION AND CONCLUSIONS

The principal results of the experiment are given in Table 17.

A cholesterol diet causes hypercholesterolemia and disturbance of the cholesterol and fat metabolism, necessary conditions for experimental atherogenesis in cockerels. Cholesterol also affects thyroid function and, by this route, the endocrinous system and general metabolism. As a cholesterol diet tends to inactivate the thyroid the so-called endocrinous factor in the mechanism of action of cholesterol obviously has an intensifying effect on atherosclerosis. It was possible to show with radioactive sulphur that cholesterol feeding caused changes in the sulphur metabolism of the mucopolysaccharides also at a site in the aorta where no atherosclerotic changes were demonstrable on gross examination. These observations warrant the assumption that cholesterol-induced disturbances in the aortic metabolism may be primary local changes in atherogenesis and that mucopolysaccharides increase only as a later consequence of degeneration.

The way in which thyroid affects atherosclerosis is a complicated process. The circumstance that the positive correlation between the serum cholesterol level and aorta atherosclerosis disappears when thyroxin and thiouracil are given suggests that the essential effect of the thyroid on cholesterol atherosclerosis is based on a mechanism other than the regulation of the serum cholesterol content. It is probable that thyroxin- and thiouracil-induced intimal lesions as well as the effects of these substances on the aortic sulphur metabolism are of significance in the development of atherosclerosis. Fluctuations in thyroid function change the thyrotrophin production of the hypophysis, which in turn has a direct influence on the connective tissue. Indeed, it is obvious that the factors regulating the metabolism and mode of reaction of connective tissue influence also the development of atherosclerosis.

TABLE 17

THE PRINCIPAL RESULTS OBTAINED FROM THE EXPERIMENT, GROUP MEANS (AFTER 6 MONTHS' DIET)

Dietary group	Aorta atherosclerosis Mean	Total serum cholesterol mg%		Uptake of S^{35} by the aorta cpm/100 mg of fresh tissue	
		Mean	± s.e.	Mean	± s.e.
Controls	0.3 [1]	97	5	156	8
Cholesterol	2.7***	556***	88	188**	8
Thyroxin	0.9 [1]	108 [2]	—	176	12
Thiouracil	1.0 [1]	129**	6	201**	13
Cholesterol + thyroxin	3.3**	395**	58	263***	14
Cholesterol + thiouracil	2.9***	1067**	224	247***	13

[1] = spontaneous atherosclerosis (arteriosclerosis)
[2] = pooled sample
*, **, *** significance level of difference from control group

It can perhaps be said that there is in the wall of the aorta an active organ whose function and reaction are dependent on changes in the general systemic factors. Thus, in atherogenesis, besides its effect on cholesterol and the fat metabolism the thyroid would also have other effects bearing on the vascular wall itself.

In these test conditions disturbances in thyroid function (thyroxin and thiouracil), tend to aggravate cholesterol-induced atherosclerosis. Considering the goitre-atherosclerosis relationship on this basis it may be permissible to suggest that goitre, whether hypo- or hyperthyroid by nature, tends to intensify atherosclerosis. These observations support the view held by Stamler *et al.* (1949) that marked hormonal inbalances may exert an intensifying effect on atherosclerosis in the presence of prerequisite diet-induced metabolism derangements.

It may be said by way of conclusion that atherosclerosis is not merely passive infiltration of fats into the wall of the blood vessel but rather an active metabolic and degenerative process influenced by many factors, *e.g.* hormones.

SUMMARY

The principal aim of the present investigation was to study the effect of thyroid function on experimental atherosclerosis in cockerels. Special attention was paid to the changes occurring in cholesterol-induced atherosclerosis in the connective tissue ground substance of the aorta wall. An endeavour was made by examining these so-called tissue factors in play in the early phase of atherosclerosis to shed light on the effect mechanism of the thyroid in atherogenesis.

The test animals were 5-week-old cockerels divided into the following dietary groups for the experiment: 1) controls; 2) ordinary diet + cholesterol 1 per cent; 3) ordinary diet + thyroxin 0.00045 per cent; 4) ordinary diet + thiouracil 0.15 per cent; 5) ordinary diet + cholesterol 1 per cent + thyroxin 0.00045 per cent; 6) ordinary diet + cholesterol 1 per cent + thiouracil 0.15 per cent.

A part of the cockerels were killed after 3 months' diet and the rest after 6 months.

The methods employed were as follows:

— The severity of aorta atherosclerosis was graded macroscopically from 0 to 4.

— The histopathology of the aorta was studied by the following methods: toluidine blue staining for metachromasia, PAS staining, Sudan IV staining for the demonstration of fats, alcian blue staining for mucopolysaccharides, Weigert's staining for elastic tissue.

— The changes occurring in the metabolism of the acid mucopolysaccharides of the aorta were studied with radio-sulphur. The S^{35} deposited in the aorta was measured by a Geiger-Müller counter and the distribution of S^{35} in the aorta was studied by contrast autoradiography.

— The distribution of mass in the aorta wall was studied microradiographically, using ultra-soft X-rays.

— The total serum cholesterol level was determined after 3 and 6 months' diet.

The thyroid preparations were stained by the Weigert-van Gieson's technique.

The principal results were:

1) Spontaneous atherosclerosis

— In the control group 16 per cent of the cockerels had spontaneous atherosclerosis after a test period of 6 months. The atheromas were characterised by a small fat content and a large amount of fibrotic tissue.

— A 6-month diet of both thyroxin and thiouracil caused intimal thickenings in the aorta which could probably be regarded as intimal arteriosclerosis. As these lesions resembled spontaneous atheromas both macroscopically and microscopically and in their location, and as they were more numerous than in the control group, it may perhaps be said that thyroxin and thiouracil displayed a tendency to intensify spontaneous atherosclerosis in cockerels.

2) Cholesterol atherosclerosis

— The accumulation of fat-containing foam cells in the intima was the earliest microscopically observed change in cholesterol-induced atherosclerosis in cockerels. Judging by the histopathologic examination, cholesterol atherosclerosis was characterised by degeneration and fatty infiltration of the intima and elastic tissue. These processes were accompanied by regeneration as connective tissue reaction in connection with which mucopolysaccharides increased in amount. Intensified mucopolysaccharide stainability was established in atherosclerotic lesions as well as increased uptake of S^{35} and a decrease in dry weight in microradiography, all of which were obviously indications of an increase in the amount of mucopolysaccharides. Histologically the fatty changes were thus primary local changes, the increase in mucopolysaccharides only secondary.

— Small doses of thyroxin seemed to increase the severity of cholesterol atherosclerosis slightly. This aggravating effect was probably based at least in part on the intimal lesions provoked by thyroxin.

— Thiouracil raised the serum cholesterol content sharply but the degree of atherosclerosis only slightly. Thiouracil probably exerted an aggravating effect on atherosclerosis by increasing the serum cholesterol content, obviously also by causing intimal lesions and possibly by provoking the increased release of thyrotrophin.

— Thyroxin- and thiouracil-feeding resulted in the disappearance of the correlation between the serum cholesterol level and the severity of atherosclerosis. It seemed obvious that the essential

effect of thyroid on atherosclerosis in cockerels was based on a mechanism other than the changing of the serum cholesterol content.

3) Sulphur exchange of aortic mucopolysaccharides

— Cholesterol diet increased significantly the deposition of S^{35} at a site in the aorta where no atheromatous changes were demonstrable macroscopically. The observation suggested that changes occur in the early phase of atherosclerosis in the mucopolysaccharide metabolism and structure rather than in the amount of mucopolysaccharides. The cholesterol-induced derangements in the metabolism of the aorta might possibly be primary changes and not lipid alterations as was assumed on the basis of the histological examination.

— Thiouracil increased significantly the deposition of S^{35} in the aorta. Both thyroxin and thiouracil increased the elevated uptake of S^{35} induced by cholesterol. It was probable that these disturbances in the metabolism of the aorta wall predisposed to atherosclerosis.

In the author's opinion, in addition to the derangement of fat metabolism, cholesterol diet caused changes in the sulphur metabolism of the aorta and tended to inactivate the thyroid, with resultant changes in the endocrinous system and general metabolism. These changes were probably associated with the genesis of cholesterol atherosclerosis.

The effect of the thyroid on atherosclerosis is complicated for in atherogenesis thyroid function obviously has — besides its influence on cholesterol metabolism — other direct effects upon the wall of the blood vessel. Some of these direct effects were indicated by the thyroxin- and thiouracil-induced intimal lesions and the disturbances in the sulphur metabolism of the aorta. Furthermore, the variations in thyroid function alter the thyrotropin secretion of the hypophysis which in turn has a direct influence on the connective tissue. Clearly, the factors which regulate the metabolism and mode of reaction of connective tissue also contribute to atherogenesis.

A permissible general conclusion perhaps is that the genesis of cholesterol atherosclerosis is an active metabolic and degenerative process which is dependent on several general and local factors such as hormones.

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